PROCEEDINGS

OF THE

ROYAL SOCIETY OF LONDON

SERIES B—BIOLOGICAL SCIENCES

VOL CXVI

LONDON
Printed and published for the Royal Society
By Harrison & Sons, Ltd., 44–47, St. Martin's Lane
Printers in Ordinary to His Majesty

February, 1935

LONDON:
HARRISON AND SONS, LTD., PRINTERS IN ORDINARY TO HIS MAJESTY
ST. MARTIN'S LANE

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SERIES B VOL CXVI

No. B 801—February 1, 1935

PROCEEDINGS
OF
THE ROYAL SOCIETY

Section B.—Biological Sciences

612.115.3

Heparin and Blood Coagulation

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(Received June 2, 1934)

The preparation of heparin from dog's liver has been described by Howell and his colleagues in a series of papers (1918, 1922, 1928). The activity of this remarkable substance is such that 1 mg preserves the fluidity of 100 cc blood for 24 hours. Howell's theory of blood coagulation is based on three properties which he ascribes to heparin. (1) Heparin has no influence on the coagulation of fibrinogen by thrombase, *i.e.*, it does not act as an antithrombase; (2) when added to plasma heparin generates from some unknown precursor in the plasma a considerable quantity of antithrombase; (3) heparin is present in blood and preserves the fluidity of circulating blood by acting as antiprothrombase.

In the following pages the actions of heparin on thrombase, thrombokinase, and prothrombase have been determined, and Howell's theory of blood coagulation has been considered in the light of these experimental facts.

The methods for the preparation of thrombase and prothrombase have been described in previous papers (Mellanby, 1931, 1933). The solution of thrombokinase was prepared by extracting a testis with water, filtering the solution and highly diluting it until the limiting dilution producing definite kinase effects had been reached. The fibrinogen solution was prepared by adding one volume of saturated Am_2SO_4 to three volumes of oxalated plasma. The mixture was spun in a high speed centrifuge tube until the precipitated fibrinogen was obtained as a compact mass at the bottom of the tube. The supernatant fluid was poured off, the tube quickly rinsed out with a little

distilled water and the fibrinogen dissolved in a volume of water equal to that of the original plasma.

The thrombase solution contained 5 mg of dried thrombase in 10 cc H_2O; the heparin solution was of the same strength. The experiments were made with a specimen of heparin (5 units per mg) supplied by Hynson, Westcott, and Dunning. Some of the results were compared with those given by a purified heparin preparation (50 units per mg). No experiments have been made with the preparation of Scott and Charles (459 units per mg) (1933, *a*).

(A) *Heparin as Antithrombase*

The capacity of heparin to act as an antithrombase has been determined on the coagulation by thrombase of (*a*) fibrinogen, (*b*) oxalated plasma, (*c*) dialysed plasma, (*d*) dialysed plasma containing varying quantities of NaCl, and (*e*) fibrinogen solutions containing varying quantities of NaCl.

(*a*) *Heparin and the Coagulation of Fibrinogen by Thrombase* -A constant quantity of thrombase was added to a fibrinogen solution containing varying quantities of heparin. The following coagulation times, given in Table I, were observed :—

Table I

Fibrinogen	H_2O	Heparin	Thrombase	Coagulation time
cc	cc	cc	cc	secs
1	0·4	0·0	0·1	5
1	0·3	0·1	0·1	10
1	0·2	0·2	0·1	20
1	0·1	0·3	0·1	50

It is evident that although heparin delays to some extent the coagulation of fibrinogen by thrombase, yet this antithrombase action is not large.

(*b*) *Heparin and the Coagulation of Oxalated Plasma by Thrombase* The above experiment was repeated except that oxalated plasma was used instead of a fibrinogen solution, Table II.

Table II

Oxalated plasma	H_2O	Heparin	Thrombase	Coagulation time
cc	cc	cc	cc	secs
1	0·2	0·0	0·1	5
1	0·15	0·05	0·1	15
1	0·1	0·1	0·1	60
1	0·0	0·2	0·1	no coagulation

A comparison of the experimental results in (*a*) and (*b*) shows that heparin acts much more powerfully as an antithrombase when added to oxalated

plasma than when added to a solution of fibrinogen in water. The results confirm Howell's observations on this action of heparin.

(*c*) *Heparin and the Coagulation of Dialysed Plasma by Thrombase*—The problem arises whether conditions exist in plasma which favour the action of heparin as an antithrombase or whether, according to Howell, heparin generates antithrombase by its action on some unknown precursor present in plasma. The small but definite antithrombase action of heparin on the coagulation of a fibrinogen solution by thrombase favours the former hypothesis.

Oxalated plasma contained in a collodion thimble was dialysed against distilled water for 6 hours, the distilled water being constantly changed. The figures given in Table III show the effect of heparin as an antithrombase when added to dialysed plasma.

Table III

Dialysed plasma	Heparin	Thrombase	Coagulation time
cc	cc	cc	secs
1	0·0	0·1	10
1	0·05	0·1	10
1	0·1	0·1	12
1	0·2	0·1	15
1	0·4	0·1	15

It is evident that the removal of dialysable substances from plasma annuls almost entirely the action of heparin as an antithrombase.

(*d*) *Heparin and the Coagulation by Thrombase of Dialysed Plasma to which varying quantities of Sodium Chloride have been added*—From the previous result it appeared that inorganic salts have a marked influence on the antithrombase action of heparin in plasma. To test this hypothesis the antithrombase action of heparin was determined on dialysed plasma to which varying quantities of sodium chloride were added, Table IV.

Table IV

Dialysed plasma	NaCl 10%	H_2O	Heparin	Thrombase	Coagulation time
cc	cc	cc	cc	cc	
1	0·0	0·3	0·1	0·1	10 secs
1	0·05	0·25	0·1	0·1	25 ,,
1	0·1	0·2	0·1	0·1	40 ,,
1	0·15	0·15	0·1	0·1	9 mins
1	0·2	0·1	0·1	0·1	No coagulation

The addition of NaCl to plasma to the extent of 1% enabled 0·05 mg of heparin to extend the coagulation time of 1 cc of plasma by 0·05 mg of thrombase from 10 seconds to 9 minutes. It appears probable, therefore, that heparin

acts as antithrombase only in the presence of an adequate quantity of neutral salt.

(e) *Heparin and the Coagulation by Thrombase of Fibrinogen Solutions containing varying Quantities of Sodium Chloride* In order to confirm this conclusion the antithrombase action of heparin on a fibrinogen solution to which varying quantities of sodium chloride were added was determined, Table V.

Table V

	Fibrinogen	NaCl 10%	H_2O	Heparin	Thrombase	Coagulation time
	cc	cc	cc	cc	cc	
I	1	0	0·4	0	0·1	5 secs
	1	0	0·3	0·1	0·1	10 ,,
	1	0	0·2	0·2	0·1	20 ,,
	1	0	0·1	0·3	0·1	50 ,,
II	1	0 025	0·375	0	0·1	5 secs
	1	0 025	0·275	0·1	0·1	25 ,,
	1	0·025	0·175	0·2	0·1	5 mins
	1	0·025	0·075	0·3	0·1	6·1,,
III	1	0 05	0·35	0	0·1	5 secs
	1	0·05	0·25	0·1	0·1	55 ,,
	1	0·05	0·15	0·2	0·1	No coagulation
	1	0·05	0·05	0·3	0·1	,,

The results confirm the conclusions deduced from the previous experiments - that heparin acts as an antithrombase only in the presence of an adequate quantity of a neutral salt. For this reason heparin influences to a slight degree only the coagulation of a fibrinogen solution containing a trace of neutral salt but acts as a powerful antithrombase when tested on oxalated plasma containing the normal quantity of neutral salt. Howell's hypothesis that heparin added to plasma generates a powerful antithrombase from some unknown precursor present in it receives a simple explanation from these experimental observations.

(B) *Heparin as Antikinase*

According to Howell heparin acts as a powerful antiprothrombase. The experimental proof of this hypothesis is difficult since prothrombase is an inactive substance. Howell shows that this assumed antiprothrombase action of heparin can be annulled by tissue extract—a fact which would appear to indicate that heparin is an antikinase. To determine the precise relation of heparin to kinase, a series of experiments have been carried out to show the influence of heparin on (a) the coagulation of oxalate plasma by kinase and

calcium salts, and (*b*) the activation of prothrombase by kinase and calcium salts. A further series of experiments have been made (*c*) to show the re-activation by kinase of an inactive thrombase heparin mixture, and (*d*) to test the hypothesis that prothrombase consists of thrombase and heparin.

(*a*) *Heparin and the Coagulation of Oxalated Plasma by Kinase and Calcium Salts*—The following experimental results, Table VI, were obtained when oxalated plasma containing varying quantities of heparin and kinase was coagulated by the addition of an appropriate quantity of $CaCl_2$.

Table VI

	Oxalated plasma	Heparin	Kinase	$CaCl_2$ (0·1 N)	Coagulation time
	cc	cc	cc	cc	′ ″
I	1	0	0·1	0·2	1 30
II	1	0·1	0·1	0·2	No coagulation
	1	0·1	0·2	0·2	2 30
	1	0·1	0·3	0·2	0 25
III	1	0·2	0·1	0·2	No coagulation
	1	0·2	0·2	0·2	,,
	1	0·2	0·3	0·2	0 30

An analysis of the figures in the groups of experiments I, II, III, indicates a precise relation between the inhibitory action of heparin and the activating properties of kinase in regard to the conversion of prothrombase to thrombase. There is no evidence that a quantitative relation exists between the heparin and the prothrombase, *i.e.*, that heparin acts as an antiprothrombase. Actually the experiments recorded do not differentiate between a possible inhibitory action of heparin on (1) the conversion of prothrombase to thrombase, and (2) the coagulation of fibrinogen by thrombase, since the tests were carried out on oxalated plasma. The following experiments show that heparin has no inhibitory action on the conversion of prothrombase to thrombase by kinase and calcium salts.

(*b*) *Heparin and the Activation of Prothrombase by Kinase and Calcium Salts*—The following mixtures, Table VII, of prothrombase, heparin, kinase, and $CaCl_2$ were made up.

Table VII

	Prothrombase	Heparin	Kinase	$CaCl_2$ (0·02 N)	H_2O
	cc	cc	cc	cc	cc
(1)	1	0	0·1	0·1	0·3
(2)	1	0·1	0·1	0·1	0·2
(3)	1	0·15	0·1	0·1	0·15
(4)	1	0·2	0·1	0·1	0·1
(5)	1	0·3	0·1	0·1	0·0

After 10 minutes the thrombase activities of these mixtures were tested on dialysed oxalated plasma. From the previous experimental results it is evident that a test for thrombase on fibrinogen in the presence of heparin must be carried out on dialysed plasma; otherwise the presence of thrombase is obscured by the antithrombase action of heparin, which is exerted in the presence of neutral salts.

Table VIII

Dialysed plasma	Mixture		Coagulation time
cc	cc		secs
1	0·1	(1)	10
1	0·1	(2)	10
1	0·1	(3)	10
1	0·1	(4)	10
1	0·1	(5)	12

The results, Table VIII, conclusively show that heparin does not prevent the activation of prothrombase by kinase and calcium salts. The inhibitory action shown in the first series of experiments (*a*) in this section was due to the fact that heparin prevents the coagulation of fibrinogen by thrombase in the presence of neutral salts, and thereby simulated the absence of activation of the prothrombase.

(*c*) *The Reactivation by Kinase of an Inactive Mixture of Thrombase and Heparin*—A mixture of thrombase and heparin may be made which does not coagulate oxalated plasma, *i.e.*, the thrombase appears to be completely neutralized by the heparin. There is, however, no union between the thrombase and heparin since the thrombase contained in such a mixture coagulates rapidly the fibrinogen contained in dialysed plasma. It is a remarkable fact, however, that a mixture of thrombase and heparin which is unable to coagulate oxalated plasma can have its coagulating activity completely restored by the addition of appropriate quantities of kinase, the quantity of kinase being in direct numerical relation to the heparin.

The following mixture of thrombase and heparin was prepared:

Thrombase	Heparin	H_2O		
2 cc	1·6 cc	0·4 cc	=	A

0·2 cc of A added to 1 cc of oxalated plasma produced no coagulation although 0·1 cc of the original thrombase solution coagulated a similar quantity of plasma in 25 seconds. Varying quantities of kinase were added to 0·5 cc of this inactive mixture of thrombase and heparin and the activities of 0·4 cc of the resulting solutions were determined on oxalated plasma after 15 minutes. The results given in Table IX were obtained.

Table IX

A	Kinase	H_2O	Coagulation time of 1 cc plasma produced by 0·4 cc of each after 15 mins
cc	cc	cc	
0·5	0	0 5	No coagulation
0·5	0·1	0 4	85 secs
0·5	0·2	0·3	45 ,,
0·5	0·4	0·1	26 ,,

It is evident that the addition of kinase to the inactive thrombase heparin mixture restored the coagulating activity of the thrombase.

(*d*) *The Hypothesis that Prothrombase consists of a Complex of Thrombase and Heparin*—The results recorded in the previous section indicate that prothrombase may consist of a complex of heparin and thrombase. On this hypothesis the activation of prothrombase depends on the neutralization of heparin by kinase with the liberation of active thrombase. To test this hypothesis the influence of $CaCl_2$ and $K_2\overline{O}x$ on the reactivation of an inactive heparin thrombase mixture were determined. Although it appears probable that Ca ions are not essential for the activation of prothrombase by kinase yet the velocity of activation is profoundly influenced by the presence of these ions.

An inactive mixture (A) of heparin and thrombase was made as in the preceding experiments. The time relations for the reactivation of (A) by (1) kinase, (2) kinase and $CaCl_2$, and (3) kinase and $K_2\overline{O}x$ were determined. The results given in Table X were obtained.

Table X

A	H_2O	Kinase		Coagulation time for 0·4 cc added to 1 cc plasma after 15 mins
cc	cc	cc		secs
0·5	0·4	0·1		24
0·5	0·3	0·1	0·1 cc $K_2\overline{O}x$ 1%	25
0·5	0·3	0·1	0·1 cc $CaCl_2$ 1%	25

It is evident that the reactivation of an inactive mixture of thrombase and heparin by kinase is not influenced by calcium or oxalate ions—a fact which negatives the assumption that prothrombase consists of an inactive complex of thrombase and heparin.

(C) *Discussion of Results*

In the previous pages three facts have been established in regard to the action of heparin: (1) it prevents the coagulation by thrombase of fibrinogen con-

tained in a neutral salt solution; (2) this inhibitory action is annulled by thrombokinase; (3) heparin has no inhibitory action on the activation of prothrombase by kinase and calcium salts.

The question arises whether heparin exists in normal blood and is responsible for the continued fluidity of blood in the vascular system of the living animal. It is clear that this hypothesis cannot be tested on mammalian oxalated blood since this blood contains a quantity of kinase from the damaged tissues and blood cells more than sufficient to annul the action of any heparin which may have been contained in it. Birds' blood, however, may be obtained by careful experimentation which shows no tendency to clot. This blood shows none of the properties of heparinized blood and may be coagulated by the addition of a trace of tissue extract. Again, since heparin does not prevent the conversion of prothrombase to thrombase, that is, the initial stage of blood coagulation, it is improbable that it is responsible for the preservation of the fluidity of the circulating blood.

There is no evidence that heparin is related to the normal antithrombase of circulating blood. This antithrombase slowly neutralizes thrombase when free in serum, but does not inhibit the coagulation of fibrinogen in plasma by the thrombase generated from the prothrombase. Further, the product formed by the slow neutralization of thrombase by the antithrombase of serum is not reactivated by the addition of kinase in a manner comparable to the instantaneous reactivation of a neutral mixture of heparin and thrombase by the addition of an appropriate quantity of kinase.

It is evident that these conclusions offer no support to Howell's theory of blood coagulation which states that (*a*) prothrombase is converted to thrombase by the action of calcium ions only, and (*b*) the fluidity of blood is preserved by the presence of heparin which acts as an antiprothrombase and thereby prevents the calcium ions from converting prothrombase to thrombase. In regard to this latter point it has been shown in a previous paper that prothrombase is slowly converted to thrombase in the absence of calcium ions; a fact which is directly opposed to Howell's hypothesis. The results put forward in this paper offer additional evidence in favour of the assumption that the conversion of prothrombase to thrombase is a function of thrombokinase and that the velocity of this reaction is profoundly modified by (1) the presence of calcium ions, and (2) the hydrogen ion concentration of the reacting medium.

The widespread distribution of heparin in the tissues, and the increase in the yield of heparin obtained from tissues after autolysis by Scott and Charles

(1933, *b*), appear to indicate that heparin is concerned with the prevention of localized thrombosis in the immediate neighbourhood of disintegrating tissue.

Summary

Heparin prevents the coagulation of oxalated plasma by thrombase. This action depends upon the neutral salt content of the plasma since heparin does not inhibit the coagulation of dialysed oxalate plasma by thrombase.

The antithrombase action of heparin is annulled by thrombokinase.

Heparin does not prevent the conversion of prothrombase to thrombase by the action of thrombokinase and calcium salts.

No evidence has been obtained that heparin acts as antiprothrombase.

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The Relation of the Parathyroid Glands to the Action of Irradiated Ergosterol

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(Communicated by Sir Henry Dale, Sec. R.S.—Received July 20, 1933
Revised May 9, 1934)

In a previous communication (Taylor, Weld, Branion and Kay, 1931) we drew attention to the similarity between the effects upon dogs of overdosage with parathormone and those induced by excessive doses of irradiated ergosterol. A large number of animals were studied and several different aspects of the overdosage manifestations of the hormone and the vitamin were compared. The rise in the serum calcium effected by large doses of either of these two substances had been sufficient alone to suggest to many investigators some fundamental relationship between their actions. The suggestion was given the colour of reality by the facts, that whichever agent was employed, the magnitude of the calcium rise and the excretory response of the kidney were found to be essentially the same. The urinary calcium is greatly increased and the volume and total acidity of the urine rises in either instance. Other features common to the actions of the two substances are the rise in the non-protein nitrogen of the blood and a great elevation of the blood phosphorus in the terminal stages of the overdosage condition. These resemblances in action we believe are not without significance in view of the fact that no other agent is known to cause a comparable combination of effects. The experiments carried out at Toronto demonstrated two other remarkable points of similarity between the actions of the two agents, namely, the closely comparable degrees of susceptibility to either substance exhibited by various species and the identical nature of the vascular changes in the gastro-intestinal tract of carnivora, whichever substance was employed.

Since the publication of our earlier paper the susceptibility to irradiated ergosterol and parathormone has been investigated in two other species— pigs and lions. Eight young pigs, just weaned, were employed.* Three were each

* These experiments were carried out in association with Dr. Hugh Branion of the Ontario Agricultural College, Guelph, Ontario.

given 2 cc doses of irradiated ergosterol (10·000 X)* by mouth daily. This amounted to 0·06 cc per kilo of body weight. The remaining animals were kept as controls. One of the treated animals died at the end of the fourth week. Bleeding from the nose and difficulty in breathing were present for a day or two preceding death. At autopsy a hæmorrhagic state of the lungs was found. The second animal died after 5 weeks of treatment. The post-mortem examination showed hæmorrhages into the lungs and gastro-intestinal tract. The third animal showed no ill effects. It gained in weight throughout the experiment and in no way appeared different from the controls. From these results the susceptibility of pigs to the overdosage effects appears to be little different from that of dogs. Other members of this species exhibited a corresponding degree of susceptibility to the effects of parathormone.

The effect of vitamin D overdosage was investigated in a lioness weighing some 200 kilos. After the administration of 0·15 cc of irradiated ergosterol (10·000 X) per kilo daily for two days the animal, which had previously been in good health, refused to eat, developed diarrhœa and appeared seriously ill. It was killed. The post-mortem examination revealed an intensely congested state of the intestinal tract. Unfortunately a specimen of blood for the determination of the serum calcium was not obtainable, but in view of the typical symptoms and autopsy findings there is little doubt that hyper-calcæmia existed. From this result it appears that this carnivorous species is highly susceptible to ergosterol overdosage. Parathormone administered to a lion caused a prompt rise in the serum calcium.†

Other phenomena common to the actions of the two substances and which suggest a close physiological association between them are the withdrawal of calcium from the skeleton, the reduction in plasma phosphatase, the deposition of calcium in the soft tissues and the production of osteitis fibrosa cystica.

As a result of the evident parallelism of the effects seen in the two over-dosage conditions, we were firmly convinced that there was an intimate physiological relationship between the parathyroid hormone and the action of irradiated ergosterol. We stated that, in our opinion, the relationship was "most probably a direct one, namely, the direct stimulation of para-thyroid tissue by the sterol." The results of a series of experiments in which irradiated ergosterol was administered to normal animals, to those upon

* 1 cc of this material contains one million international rat units.

† The experiments were undertaken with Mr. John A. Cambpell, B.V.Sc., Curator of the Riverdale Zoo, Toronto.

which the ordinary thyroparathyroidectomy was performed, and to those which had undergone an assumedly complete removal of parathyroid tissue from the neck, influenced us in coming to this conclusion. It was then stated, however, that in view of the variability in the response of different animals to parathyroidectomy and of the impossibility of knowing the amount of unremoved parathyroid tissue, the results of a much larger number of experiments would be required before this method of attacking the problem could be expected to give a conclusive answer. We therefore depended more on the indirect evidence afforded by a comparison of the two sets of effects already cited in forming our opinion concerning the mode of action of irradiated ergosterol.

Among the other possible explanations which we discussed was first, that parathormone and vitamin D had an action in common upon some organ or tissue which in turn was responsible for the chracteristic effects. There is at present no evidence to offer for or against such an hypothesis. A second possibility was that hypercalcæmia was the only effect common to both agents and that the other manifestations of overdosage were consequent upon the high serum calcium. From the results of the experiments of others and ourselves there was little to indicate that hypercalcæmia itself would produce the more severe manifestations of overdosage in dogs. This point, however, will be considered later.

Dale, Marble, and Marks (1932) have recently reported a series of experiments in which the toxic effects of crystalline vitamin D (calciferol) were studied upon normal dogs and upon those in which a simple parathyroidectomy or more extensive operations for the removal of parathyroid tissue had been performed. They were unable to demonstrate that parathyroidectomized animals responded less readily than did normal animals, and stated that their results "lend no support to the suggestion that vitamin D in excessive doses acts by promoting the secretion of the parathyroid hormone, or by rendering an organism more responsive to its action."

Though Dale, Marble, and Marks have come to the foregoing conclusion it will be pointed out later that a significant difference between the response in their two groups of animals did, nevertheless, exist. The reason that a greater difference does not appear may possibly be accounted for by assuming that the calcium-raising property of calciferol is must greater than that of the irradiated ergosterol (10·000 X) employed by ourselves. It is quite conceivable that any difference in the degree of intensity of the reaction in parathyroidectomized as compared with that in the normal animals which

might be evident when irradiated ergosterol of a certain hypercalcæmic and toxic power was employed might, on the other hand, become more or less obscured if the animal were treated with a preparation possessing a much greater power. Our reason for considering this possibility was the extraordinarily prompt manner in which their animals responded to the administration of the vitamin. Dale and his associates in their experiments upon normal dogs obtained a rise in the serum calcium of as much as 5 mg within 24 hours, and a rise of nearly 9 mg in 48 hours. In none of our previous experiments did we observe any rise in the serum calcium of normal animals receiving maximal doses of irradiated ergosterol (10·000 X) before the lapse of 48 hours. Even then, the rise amounted to no more than 1 or 2 mg per cent., and one of 9 mg per cent. has not been observed before from 60 to 90 hours. In our experiments the usual response observed during the first 24 hours of the administration of irradiated ergosterol was a fall in the serum calcium of from 1 to 2 mg. Since the publication of the paper by Dale, Marble and Marks we have searched our records, which include experiments upon some 50 adult dogs treated with irradiated ergosterol, and find that in 25 dogs, on which daily determintions of the serum calcium were made, in none did the calcium curve show the features observed by Dale and his associates. This discrepancy between their results and our own pointed to a great difference between the calcium-raising power of calciferol and the preparation employed by us. Upon the basis of rat units, the quantity of the vitamin (calciferol) administered by Dale, Marble and Marks to their animals was some three to four times greater than the quantity used in our experiments. We therefore gave a number of animals irradiated ergosterol (10·000 X) in a dosage equivalent in rat units to the doses of calciferol which they employed. Even so, no rise in the serum calcium was observed until after the lapse of 24 hours. We then obtained a sufficient quantity of calciferol for the treatment of two dogs. Though maximal doses were administered neither animal showed a rise within 24 hours and the hypercalcæmia did not reach its final height until after 48 hours. We are unable to account for the difference between these results and those of Dale and his associates.

The Relation of Hypercalcœmia to the Vascular Changes in the Mucosa of the Gastro-intestinal Tract

The remarkable parathormone-like effect of irradiated ergosterol upon the gastro-intestinal mucosa which was pointed out in our earlier paper strengthened

us in the opinion that the actions of the hormone and the vitamin were in some way interdependent. The effects in the two instances might nevertheless be simply the result of the hypercalcæmia. Dale and his associates, probably as a result of Collip's experiment quoted below, have come to the conclusion that the gastro-intestinal features are the result of an excess of calcium in the blood and tissues. In previous experiments we were unable to establish a constant relationship between these two effects. In puppies, for instance, death may occur after the hypercalcæmia, resulting from vitamin D overdosage, has existed for 48 hours or so, although no congestion is present in the gastro-intestinal tract.

Collip (1926) reported similar effects to those produced by parathormone overdosage following the injection of calcium combined with *acid sodium* phosphate. The effect was not produced when calcium and the *alkaline* phosphate were employed. In vitamin D or parathormone overdosage the gastro-intestinal signs appear before any notable rise in blood inorganic phosphorus occurs. Others have reported experiments in which calcium salts were infused without ill effects, but these experiments were carried out over periods of insufficient duration to decide the question, since the gastro intestinal features of parathormone or ergosterol overdosage do not make their appearance until after the hypercalcæmia has persisted for several hours. We considered it desirable to study the effects of the injections of calcium into the blood stream over periods of comparable duration. With this end in view the following experiments were undertaken.

Calcium chloride, lactate or gluconate as a 0·3%–0·6% solution of calcium in distilled water, or in a gum or a gelatin solution was injected by means of a Robinson continuous perfusion apparatus into the femoral vein. Blood samples were taken from the opposite femoral at hour intervals for calcium determinations. In all our later experiments no general anæsthetic was employed. The vein was opened and the cannula inserted after anæsthetizing the overlying tissues with novocaine. To prevent any sudden movement which might dislodge the cannula from the vein the animal's limbs were fastened to a table in a comfortable position and the body held down by tapes fastened to a canvas jacket clothing the chest and abdomen. The animal after a little training lay quietly throughout the experiment. The quantities of calcium in the solutions employed were determined by analysis. From 3 mg to 20 mg of calcium were injected per kilogram weight per hour, the quantity being varied in accordance with the level of the serum calcium during the preceding hour. An endeavour was made to maintain the concentration of calcium in the serum

at from 15 to 20 mg per cent. This we found very difficult to do, since the calcium disappeared from the blood stream with great rapidity. In several experiments, however, we were able to maintain a high level of hypercalcæmia for a number of hours and in some from 20 to 24 hours. When the lactate or gluconate was employed a condition was never found post-mortem at all resembling parathormone or vitamin D overdosage, the gastric and intestinal mucosa showing no significant departure from the normal.

In one experiment in which calcium chloride was used some suggestive patches of hypercalcæmia of the mucosa were found, but these did not approach the appearance seen after overdosage with parathormone or irradiated ergosterol. The protocol of dog 131, which received more than 600 mg of calcium over a period of 22 hours, is given in Table I. Dog 180 was given 378 mg of calcium, in the form of the gluconate, subcutaneously over a period of 6 hours. Little elevation of the serum calcium resulted. A week later continuous infusion of calcium gluconate was performed. The animal received a total of nearly 1400 mg over a period of 13½ hours. The serum calcium rose steadily and reached the extraordinary height of 60 mg per 100 cc. Nothing abnormal was observed after death in the gastro-intestinal tract (Table II).

These experiments have the possible objection that they did not last for a sufficient length of time to permit the characteristic effects to develop. The following results, however, indicate that hypercalcæmia *per se* is not the cause of the toxic symptoms in overdosage with irradiated ergosterol or parathormone.

Animal No. 247 was given very small daily doses of parathormone (0·05 cc per kg) for a period of 4 days. These were gradually increased over a period of some weeks. As will be seen in Table VI no definite rise in the serum calcium resulted until the end of the second week. A gradually increasing hypercalcæmia occurred after this and reached the level of 18·6 mg per cent. about a month from the commencement of the experiment. The animal appeared quite well until after the final increase in dosage, when dullness and loss of weight became noticeable. Nevertheless, it did not succumb until over a month after hypercalcæmia was first noticed; loss of appetite, vomiting and diarrhœa were absent throughout the course of the experiment and for nearly 3 weeks a hypercalcæmia of between 18 and 19 mg per cent. was maintained. Post-mortem, the gastro-intestinal tract showed moderately severe signs of parathormone overdosage. The result of this experiment is interesting in view of the fact that, in the dog, parathormone when given repeatedly in unaltered dosage not infrequently loses its hypercalcæmic effect.

Table I—Dog No. 131. Weight 10 kg. Continuous infusion of calcium lactate

Date and hour	Quantity of calcium infused	Serum calcium, mg per cc	Remarks
Nov. 24, 1932.			
12.10 p.m.	Infusion of calcium lactate solution (3 mg per cc) commenced	11·5	
1.10 ,,	Animal has had 20 cc of solution (60 mg Ca) during past hour	11·7	
2.10 ,,	Strength of solution increased to 6 mg per cc. 5 cc of this solution (30 mg Ca) given in past hour	10·0	
3.10 ,,	30 mg. Ca given in past hour	11·8	
4.10 ,,	30 mg ,, ,,	12·1	
5.10 ,,	30 mg ,, ,,	—	
6.10 ,,	30 mg ,, ,,	17·9	
7.10 ,,	30 mg ,, ,,	17·4	Dog restless.
8.10 ,,	30 mg ,, ,,	15·7	Very restless, resp. 64, pulse 150.
9.10 ,,	60 mg ,, ,,	15·0	
9.25 ,,	—		Resp. 32, pulse 156, temp. 38.
10.10 ,,	30 mg Ca given in past hour	16·3	
11.10 ,,	60 mg ,, ,,	17·6	Vomited, resp. 22.
12.10 a.m.	30 mg ,, ,,	15·6	Restless, rapid respiration.
1.10 ,,	30 mg ,, ,,	17·5	Drank some water, but vomited.
2.10 ,,	No Ca given in last hour	15·4	
3.10 ,,	18 mg Ca given in last hour	15·6	
4.10 ,,	No Ca given in last hour		
5.10 ,,	30 mg Ca given in last hour	17·0	Movement of bowels. Pulse 190.
6.10 ,,	30 mg ,, ,,	—	Fine tremor of muscles.
7.10 ,,	18 mg ,, ,,	15·0	Animal very quiet.
8.10 ,,	No Ca given in last hour	—	
9.10 ,,	30 mg Ca given in past hour	10·9	Vomited, resp. 27, pulse 128.
10.10 ,,	60 mg ,, ,,	12·1	Tremors of limb muscles.
11.10 ,,	No Ca given in past hour	—	Died 11.15. Post mortem. Heart muscle relaxed, right ventricle dilated. Lungs showed a few small patches of congestion. Mucosa of stomach and duodenum show nothing abnormal.
	Total quantity of calcium infused, 666 mg.		

The Effect of Atropinization upon the Development of the Gastro-intestinal Features of Overdosage

The appearance of the gastro-intestinal mucosa of an animal killed by parathormone, or irradiated ergosterol, suggested the possibility of a neurogenic factor being concerned. In the course of experiments in which this possibility was under investigation it was found that full atropinization of the animal greatly ameliorated or entirely prevented the development of the characteristic

Table II—Dog No. 180. Weight 8·5 kg. The subcutaneous injection of a solution of calcium gluconate (6 mg per cc) over a period of 6 hours, followed a week later by the continuous intravenous injection of a similar solution over a period of $13\frac{1}{2}$ hours.

Date and hour	Quantity of calcium injected	Serum calcium, mg per cc	Remarks
April 5, 1933.			
11.00 a.m.	Subcutaneous injection of 42 mg Ca	13·0	—
12.00 noon	,, ,,	—	—
1.00 p.m.	,, ,,	12·0	—
2.00 ,,	,, ,,	—	1.20 p.m. vomit.
3.00 ,,	,, ,,	12·4	—
4.00 ,,	,, ,,	—	4.40 p.m. vomit.
5.00 ,,	,, ,, 84 ,,	14·6	—
	Total quantity of Ca injected 336 mg		
April 6.			
10.00 a.m.	—	11·4	—
April 12.			
11.00 a.m.	Continuous infusion commenced	11·0	—
12.00 noon	60 mg Ca given during past hour	15·6	—
1.00 p.m.	60 mg ,, ,,	20·0	—
2.00 ,,	90 mg ,, ,,	—	—
3.00 ,,	No Ca given in past hour	—	—
4.00 ,,	,, ,,	—	—
5.00 ,,	120 mg Ca given during past hour	24·2	—
6.00 ,,	145 mg ,, ,,	—	—
7.00 ,,	120 mg ,, ,,	46·6	—
8.00 ,,	120 mg ,, ,,	—	—
9.00 ,,	145 mg ,, ,,	—	—
10.00 ,,	145 mg ,, ,,	61·0	—
11.00 ,,	180 mg ,, ,,	—	—
12.00 ,,	120 mg ,, ,,	58·6	—
12.30 a.m.	60 mg Ca given during past half-hour. Total quantity of Ca infused 1345 mg.	—	Died. Post-mortem. Nothing abnormal in gastro-intestinal tract.

vascular changes of parathormone overdosage. Atropinization, on the other hand, had no effect upon the development of the hypercalcæmia. The effect of atropine upon the vascular changes has been investigated in some seven animals. In none did the gastro-intestinal tract present the usual appearance of parathormone overdosage. The duodenal mucosa in all cases was quite pale when adequate doses of atropine were administered. We have found that dogs quickly become tolerant to this drug and it has been necessary to increase the dosage each day. In order to prevent the congestion of the gastric mucosa very large doses had to be administered. Even so, though the the mucous membrane of the stomach for the most part was quite clear, a few small patches of congestion were usually evident. The dose of parathormone given to these animals was several times greater than that which will produce the usual effects in an unatropinized animal. The hormone was administered subcutaneously at 3-hour intervals throughout the day and the

otal dose amounted to from 500 to 650 units. The greatest dose of atropine ıdministered to these animals was 0·6 mg per kg. This was given with each lose of the hormone. In Table III are shown the details of an experiment upon log 172.

Table III—Dog No. 172. Weight 27·0 kg. Treated with parathormone and atropine

Date and hour	Quantities of parathormone and atropine given subcutaneously		Serum calcium, mg per cc	Remarks
Mar. 18, 1933.				
12.00 noon	2·5 cc parathormone.	5·4 mg atropine	11·9	..
4.00 p.m. ...	2·5 cc ,,	5·4 mg ,,	—	..
11.00 ,, ...	2·5 cc ,,	5·4 mg ,,	--	
Mar. 19.				
8.00 a.m. ..	2·5 cc ,,	8·0 mg ,, ...	19·2	..
11.00 ,,	2·5 cc ,,	8·0 mg ,,		.
4.00 p.m.	2·5 cc ,,	8·0 mg ,,		.
11.00 ,,	2·5 cc ,,	8·0 mg ,,		No vomiting or diarrhœa at any time during treatment.
Mar. 20.				
10.00 a.m.	2·5 cc ,,	10·8 mg ,, ..	18·6	
12.00 noon	2·5 cc ,,	10·8 mg ,,		
4.00 p.m.	2·5 cc ,,	10·8 mg ,,	.	
Mar. 21.				
10.00 a.m. .	2·5 cc ,,	10·8 mg ,, ...	14·0	
12.00 noon . .	2·5 cc ,,	16·0 mg ,,		
4.00 p.m. ..	2;5 cc ,,	16·0 mg ,,	.	
Mar. 22.				
8.00 a.m.	—		.	Found dead. Stomach mucosa greyish colour. A few small spots of congestion in fundic region. Rest of gastric mucosa quite free from congestion or ecchymosis. Intestine quite pale and normal in appearance.

Total dose of parathormone 650 units.

The effect of atropinization upon the gastro-intestinal features of overdosage with irradiated ergosterol was as a rule less marked. The intensity of the vascular effects was reduced but in most experiments was not entirely abolished. This may be due to the fact that, unlike that of parathormone, the action of irradiated ergosterol is continuous and is more difficult to overcome by atropine, which must of necessity be administered at intervals. The effect of atropine upon the vascular changes was most pronounced in dog 162 (Table IV). In this animal 10 times the dose of irradiated ergosterol which produces the characteristic effect was given. The serum calcium rose to a very high level and the animal lived for 14 days. After death the mucous membranes of the

stomach and intestine were uniformly quite pale. Not even the smallest area of congestion was visible.

The foregoing experiments, in which the serum calcium was raised by calcium injections, or by parathormone and vitamin D administration, to atropinized animals suggest that the gastro-intestinal features characteristic of over-dosage by either of these substances is not simply the result of the high concentration of calcium in the blood and tissues. The fact that atropine abolishes or reduces the intensity of the vascular changes suggests that the gastro-intestinal features have a neurogenic basis.

Table IV—Dog No. 162. Weight 8·5 kg. 0·4 cc of irradiated ergosterol (10·000 X) per kg given three times daily. 1·7 mg atropine given at 3-hour intervals throughout the day.

Date and hour	Serum calcium, mg per cc	Remarks
February 21, 1933	12·8	—
February 22—		
10.00 a.m.	12·4	Very nervous and excitable, apparently effect of atropine.
5.00 p.m.	13·4	—
February 23	14·0	Quieter.
February 24—		
10.00 a.m.	16·4	—
5.00 p.m.	17·6	—
February 25	20·0	—
February 26	21·9	—
February 27	21·8	Dull and depressed. Some twitchings of limb muscles.
February 28	23·8	Dull. Shivering.
March 1	27·0	Little change in condition.
March 2	25·2	Weak and dull.
March 3	25·8	”
March 4	—	Found dead. Post-mortem. Intestine and stomach quite clear. No congestion of mucosa.

Discussion

In order to explain our results, which appeared to show that parathyroidectomized animals were relatively resistant to the action of irradiated ergosterol, Dale, Marble and Marks offer as an alternative theory one which does not involve any essential physiological relationship between the hormone and the vitamin. They believe that since parathyroid removal lowers the serum calcium below the normal level and irradiated ergosterol causes a rise above this level, the effect of the latter substance upon the serum calcium after parathyroidectomy is determined by the algebraic sum of these two opposing influences. For this reason hypercalcæmia, it is supposed, can be more easily produced in normal than in parathyroidectomized animals.

The principle involved in such a simple explanation, obviously, should be of general application. That is, any agent which raises the serum calcium should cause a greater degree of hypercalcæmia if administered to an animal, whose serum calcium to start with was normal, than if administered to one with a

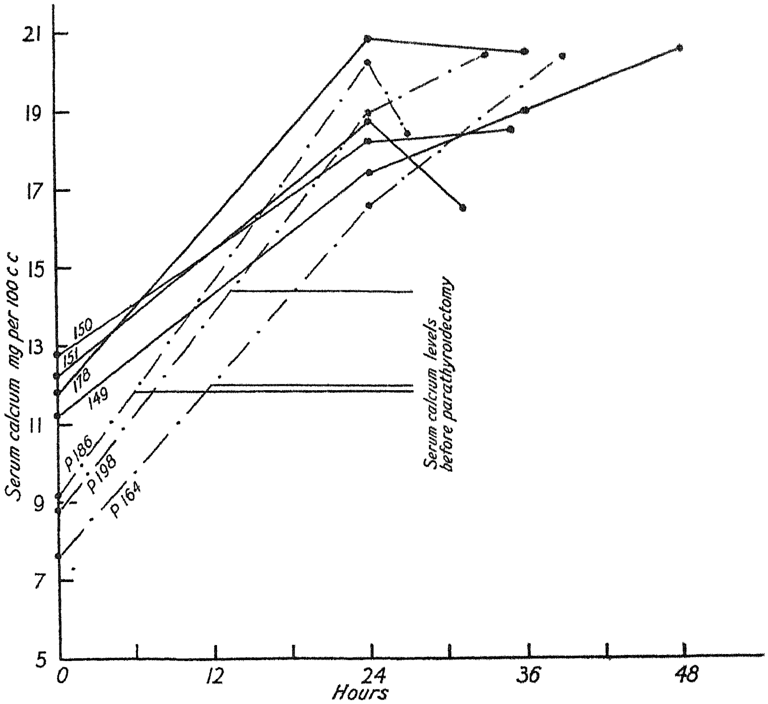

FIG. 1—The serum calcium curves of parathyroidectomized animals receiving large doses of parathyroid extract compared with those of normal animals treated similarly. Continuous lines = normal animals. Broken lines = parathyroidectomized animals. Compare these curves with those shown in fig. 2. As the quantity of parathormone per animal may be as important as the amount per kg in computing dosage, the following dosage was employed :—

Dog	Units of parathormone four times daily	Per kg	Dog	Units of parathormone four times daily	Per kg
149	20	3·1	P 198	30	2·8
150	40	7·3	P 164	40	2·2
151	40	8·0	P 186	60	2·4
178	40	2·1			

falling serum calcium or with hypocalcæmia. This is not so however, since neither the intravenous injection of calcium nor the administration of parathormone, which like irradiated ergosterol withdraws calcium from the bone, raises the serum calcium more promptly or to a higher level in the normal than

in the parathyroidectomized animal, see fig. 1. As against this, an analysis of the figures given in the experiments of Dale, Marble and Marks shows that irradiated ergosterol does cause a more prompt increase of the serum calcium of normal animals than of those upon which parathyroidectomy had been performed. These results are shown in fig. 2. In coming to the conclusion that parathyroidectomy was practically without effect upon the action of calciferol, Dale, Marble and Marks apparently only considered the final calcium levels, and neglected the figures for the first 24 hours and for 48 hours, which do certainly indicate that parathyroidectomy had a restraining influence

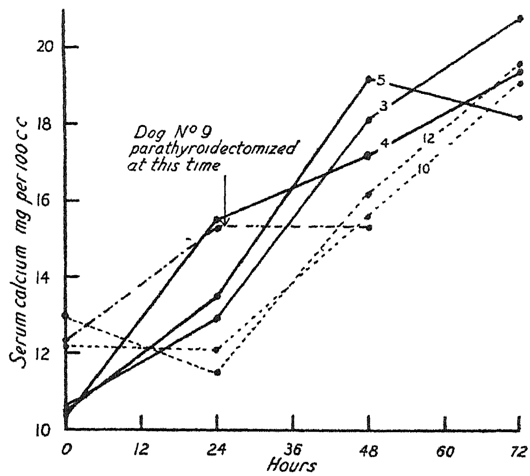

FIG. 2—The serum calcium curves of parathyroidectomized animals treated with calciferol compared with those of normal animals receiving equivalent doses of the vitamin. Drawn from data reported by Dale, Marble and Marks. Continuous lines = normal animals. Broken lines = parathyroidectomized animals. Compare these curves with those shown in fig. 1.

upon the calcium-raising effect of the vitamin. After the first 24 hours the rate of rise of the calcium of the serum is similar in both groups of animals. The calcium-lowering effect of parathyroidectomy certainly would persist throughout the second and third days and, were the explanation offered by Dale and his colleagues correct, its antagonism to the calcium-raising effect of calciferol should still be reflected at this time in the slope of the calcium curve, which is not so. These results therefore conform more readily to the conception of an intimate parathyroid-vitamin relationship. The failure of the curve to rise within the first 24 hours is explained best by postulating that some hours are required for the stimulation or possibly for the actual hyperplasia of remnants of parathyroid tissue.

It may be noted that in preparing fig. 2 from Dale's figures, the serum calcium levels of the operated animals 7 or 8 hours after parathyroidectomy have been omitted. This is done because no corresponding values are given in the control curves. Consequently one cannot determine the separate effect of either parathyroidectomy or the administration of calciferol upon the serum calcium level at this time.

We wish to refer to the final experiment of Dale and his colleagues which was performed in an effort to provide a decisive answer to the question of the responsiveness of an animal to irradiated ergosterol overdosage after parathyroid deprivation. The result of this experiment is capable of a different interpretation from the one which they have placed upon it. Complete eradication of all parathyroid tissue was attempted in three animals. The operation involved splitting the sternum and extirpation of the thymus. Two of these animals died under the anæsthetic; the third survived the operation for some 45 hours. In view of the severity of this operation and the mortality attendant upon the previous two attempts made to remove the thymus, we fail to see how it is possible to ascribe the death, as Dale does, to calciferol. There is no evidence that parathyroidectomized animals are more susceptible to the vitamin than are normal animals which survive these doses for from 70–100 hours. The serum calcium in this animal rose only 3·7 mg per cent, which is only a fraction of that which would be expected in normal animals. Even this small rise cannot be attributed properly to the calciferol, for we have accumulated considerable evidence that any severe tissue trauma may cause a transient rise of the serum calcium. In the course of the past few years the serum calcium has been followed in animals which have been subjected to various types of experimental procedure, and it has been our experience to find a rise of several milligrams under most conditions in which severe injury had been involved. In experimental intestinal obstruction, for instance, it is not unusual to observe a rise of 3 or 4 mg per cent when the animal becomes seriously ill. The calcium rise was shown not to be due to blood concentration. At one time we were interested by this response to obstruction of the bowel until we concluded that it was not a specific effect but occurred under other circumstances. Evisceration in dogs produces a rise in the serum calcium of several milligrams within a few hours. Adrenalectomy causes a rise in both dogs and cats which cannot be accounted for by blood concentration and is not, we believe, a specific effect of adrenal (cortical) deficiency, but is simply associated in some unexplained way with the moribund state. In cats we have repeatedly encountered a pronounced serum calcium rise after decapita-

tion and skinning of the body. Even after relatively simple abdominal operations it is not uncommon to find the serum calcium elevated 1 or 2 mg above the normal for the subsequent 24 hours (Table V).

Apart from other considerations the striking resemblances which have been observed between the effects of parathormone overdosage and those resulting from an excess of irradiated ergosterol weigh heavily, in our opinon, in favour of a close physiological relationship underlying the actions of these two substances. It seems scarcely possible that the several points of similarity between their effects are merely coincidental and that both substances act directly and independently upon the calcium stores of the body. There is much more than a suggestion that these substances are in physiological communion. Though a final judgment concerning the *nature* of the relationship must be reserved until fresh experimental work has made the way clearer, the balance of evidence at the present time is, we believe, in favour of a direct action of vitamin D upon parathyroid issue. This question can only be tested directly either by a study of the effects of vitamin overdosage before and after operations for the extirpation of parathyroid tissue, or by the examination of parathyroid tissue following the administration of the vitamin to normal animals for hyperplastic changes, or other evidence of increasing glandular activity. The difficulties in the way of the former mode of attack have been mentioned. Nevertheless, the results of the experiments of Hess, Weinstock and Rivkin (1929) with irradiated ergosterol are highly significant. These authors performed a number of experiments and found that whereas hypercalcæmia could be induced in monkeys and dogs with intact parathyroids, the serum calcium could not be raised above the normal after parathyroidectomy. In a later series of experiments, however, Hess, Weinstock and Benjamin (1930) found that hypercalcæmia could be caused in parathyroidectomized animals by increasing the dose of irradiated ergosterol by from $2\frac{1}{2}$ to 20 times above and used in the previous series. These experimental results disclose clearly that the susceptibility of animals to the effects of irradiated ergosterol is lowered by even the ordinary operation of thyroparathyroid extirpation.

It should be mentioned, however, that the action of ergosterol appears to differ in one respect from the action of parathyroid extract. When given to dogs in overdosage, though not when given in small doses (Taylor and Weld, 1932, *b*), the vitamin depresses the excretion of calcium by the intestinal epithelium. This effect has not been observed as a result of parathormone administration. Pugsley (1932) has demonstrated this difference in the actions of the two substances for rats, and in one experiment in which we investigated

Table V—Showing the effect of several types of severe injury upon the serum calcium. The results of 47 out of a total of 63 experiments are given. Of the remaining 16 experiments 5 showed a rise of 0·5 mg or less in the serum calcium; in 7 there was no change, and in 4 a slight fall was observed. In cats evisceration (removal of gastro intestinal tract) produces little or no elevation of the serum calcium.

Animal		Serum calcium mg per cent Normal	Serum calcium mg per cent After injury	Nature of injury
Dog	15	12·0	15·0	Intestinal obstruction
	17	12 0	16·1	
	20	11·8	14·3	
	21	11·3	16·1	
Cat	33	9·6	11·0	Suprarenalectomy
	36	10·0	11·0	
	40	10·3	11·0	
	41	10·4	11·2	
	44	10·4	11·1	
	48	11·0	12·8	
	49	9·5	11·8	
	66	9·2	13·2	
	72	9·2	11·8	
	74	10·0	10·9	
Dog	2	10·3	12·2	
	3	12·0	13·0	
	4	11·0	14·8	
	5	12·0	15·6	
	6	12·0	13·3	
Cat	53	10·8	12·6	Adrenal vessels ligated
	56	10·3	13·4	
	80	10·4	14·2	
Cat	24	10·8	12·0	Evisceration and suprarenalectomy
	27	10·6	12·6	
	29	11·0	12·4	
	30	10·8	13·0	
	31	10·2	13·2	
	45	10·0	11·1	
	46	10·7	14·4	
	47	10·4	12·4	
	50	10·4	12·6	
Dog	9	10·8	11·6	Suprarenalectomy 24–48 hours after parathyroidectomy
	10	5·5	7·9	
	15	5·3	6·2	
	16	9·6	10·6	
Cat	62	8·7	10·2	
	75	10·2	12·0	
	79	9·6	10·6	
Dog	1 E	13·1	21·6	Evisceration
	2 E	11·8	14·3	
Cat	1 S	10·3	13·1	Decapitation and skinning
	2 S	8·7	9·9	
	3 S	11·3	15·5	
	6 S	10·2	12·0	
	7 S	10·4	15·0	
	9 S	10·3	12·2	
	0 S	9·1	10·7	

the question upon a dog no diminution in the fæcal calcium was observed as a result of parathormone overdosage. Yet, in any attempt to arrive at a decision concerning the existence of a physiological relationship between parathormone and irradiated ergosterol through a comparison of their effects, two points which we believe worthy of some consideration are usually disregarded. In the first place, the hormone secreted into the blood stream by living parathyroid cells is probably far from being identical with the commercial extract. Indeed, in view of the method of preparation of the latter,

Table VI—Increasing Doses of Parathormone. Dog No. 247

Date	Serum calcium mg %	Parathormone cc daily	Remarks
February 15	11·5	0·05	5 kg
,, 16	11·4	(4 days)	Mature dog
February 19	—	0·10	—
,, 24	12·0	(7 days)	—
February 26	11·0	0·20	—
March 1	—	(4 days)	—
March 2	—	0·30	—
,, 6	12·2	(6 days)	—
March 8	—	0·50	—
,, 9	13·6	(14 days)	—
,, 12	15·8		Appearance normal
,, 19	18·6		,,
,, 21	18·8		—
March 22	—	0·75 (21 days)	Some depression, beginning to lose appetite
,, 26	19·0		Miserable, losing weight
April 7	17·5		No vomiting or diarrhœa. Blood inorganic phosphorus = 3·0 mg %.
,, 11	—		Vomited food, no blood, no diarrhœa, appetitite fair
,, 12	19·0		Dead

Autopsy shows congestion and ecchymoses throughout small intestine, with bloody intestinal content. Gastric mucosa only mildly congested. Lungs hæmorrhagic.

it would be strange if the natural hormone and the extract were precisely similar in their actions. In the second place, a theory which attributes the action of massive doses of vitamin D to some relationship with the function of the parathyroids, does not necessarily imply the exclusion of some additional mode of action. It is not inconceivable, for instance, that the vitamin may act also upon some other structure or tissue, and a supplementary action of this nature may account for some minor differences between its action and that of the injected parathyroid extract. Indeed, the effect of small doses of the vitamin in lowering the serum calcium and in curing rickets may possibly be

an example of this (Taylor and Weld, 1932, *a*, and 1933). For these reasons outstanding points of similarity are of greater evidential value for, than are minor points of dissimilarity against, the conception that the action of excess of the vitamin is dependent upon parathyroid function. So, while in the effect of irradiated ergosterol upon the fæcal calcium a divergence between the actions of the two substances is apparent, this is not a sufficient warrant for a decision against a parathyroid-vitamin relationship. It should be remembered that, in the main, the effects of the extract and the vitamin upon calcium excretion are the same, both increasing the excretion of calcium through the kidney. Neither increases the excretion through the bowel in dogs or in human subjects, and it is only in excessive dosage, and in the later stages of the resulting condition, that irradiated ergosterol shows its depressant action upon the calcium excretion through the intestinal mucosa.

We wish to extend to Professor C. H. Best our cordial thanks for the interest he has shown in these researches.

Summary

1—The gastro-intestinal features of overdosage with parathormone and irradiated ergosterol are not the result of the hypercalcæmia.

2—Evidence has been given which suggests that these features have a neurogenic basis.

3—It has been pointed out that the actions of other agents which elevate the serum calcium is no less in a parathyroidectomized than in a normal animal. The theory offered by Dale and his colleagues to explain the relative resistance of parathyroidectomized animals to overdosage with irradiated ergosterol is therefore not supported.

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Physico-Chemical Studies of Complex Organic Molecules
III—*Surface Properties of Concentrates of Vitamin A*

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(Communicated by T. M. Lowry, F.R.S.—Received March 21, 1934)

At present little is known of the surface properties of vitamin A or of carotene. Such knowledge can be useful in determining the shape, size, and chemical structure of these molecules. It is also possible that a knowledge of the surface behaviour of the molecules may help to throw light on the mechanism by which they are carried and are effective in the animal body.

The first part of this paper describes the properties of a surface film of a concentrate of vitamin A at a water/air interface. This concentrate spread readily on clean water to form an insoluble film of the liquid expanded type; the film remained liquid under high pressures and the general behaviour resembled that of a long chain alcohol. The surface film was oxidized by air with a diminution in the surface area. Carotene did not spread on water.

The second part gives some quantitative measurements of the adsorption of vitamin A and carotene on a number of different solid surfaces. The amount of vitamin A adsorbed depended to a marked degree upon the nature of the solvent. It was much more readily adsorbed from a non-polar solvent such as cyclohexane than from a polar solvent such as chloroform. The adsorbed vitamin could be recovered practically unchanged from the surface. Carotene was more strongly adsorbed on the surfaces than vitamin A.

Preliminary experiments indicated that the pulped liver of an "A-free" rat can take up vitamin A, but not carotene, from a solution in cyclohexane.

Surface Films of Vitamin A Concentrate*

Preliminary measurements of the surface tension of a dilute solution of a vitamin A concentrate† were made by the drop weight method. There was no indication that the vitamin possessed any abnormal surface properties.

* The experimental work in this section was carried out by Mr. D. J. C. Gamble and Mr. H. F. Kenyon.

† A British Drug Houses concentrate $\frac{1\%}{1\text{ cm}}\ E_{3280} = 500$.

The surface tensions of cyclohexane and of alcohol were not affected by dissolving in them small amounts of the concentrate, showing that the vitamin does not accumulate in the surface layers of these solvents. Adding a drop of a concentrated solution of the vitamin to water lowered the surface tension by 7%, but this does not mean very much, since the vitamin formed an in soluble film and was not, therefore, in true solution.

The surface pressure exerted by the film was measured on a Langmuir trough. A vitamin concentrate* was dissolved in air-free benzene and stored under an atmosphere of nitrogen. A few drops of the solution were added to a surface and measurements of surface pressure made as soon as possible. In fig. 1 the pressure in dynes per sq cm is plotted against the area in square Angstrom units occupied by a milligram of the concentrate. Curve 1 shows the pressure exerted by the film as it was compressed. This curve was not reversible, and it was evident that a slow change, accompanied by a diminution in area was occurring in the film during the time of compression. Curve II is for a film which had been left on the surface for 30 minutes before compression. The surface area has decreased considerably. Curve III is for a film which was spread on a dilute solution (0·01%) of potassium permanganate in N/100 H_2SO_4. This drastic oxidation diminished the surface area to about one-third of its original value. The surface was also diminished by bubbling oxygen through the benzene solution of the vitamin before spreading it on the surface, but this oxidation was very much slower. The film was then spread on a solution of 1% hydroquinone in order to reduce the amount of surface oxidation. The pressure/area curve is plotted as curve IV. The curve was now reversible and reproducible showing that the oxidation had been checked. The surface area agreed approximately with that of a very freshly-spread film on water.

Since pure vitamin A has not yet been prepared, and its molecular weight is not known with certainty, it is not possible to calculate the area of the molecule with any precision. The molecular weight from Karrer's formula $C_{20}H_{30}O$ is 286, the experimental value obtained by measurements of diffusion is 330 (Bruins, Overhoff, and Woolf (1931)). If we accept the latter value and assume further that all the concentrate is vitamin A, the molecular area can be calculated.

The film first exerted an appreciable pressure when compressed to an area of 12,000 sq cm per milligram ; the area per molecule at this point of inflection

* A British Drug Houses concentrate $\frac{1\%}{1\ \text{cm}}$ $E_{3280} = 1000$. This was 63% of the strength of the best preparation of Carr and Jewell ('Nature,' vol. 133, p. 92 (1933)).

on the above assumption would be 65 A². When the area was further decreased the pressure rose rapidly and almost linearly until it reached a value of 26 dynes per cm, where a second break occurred in the curve. The area per mole-

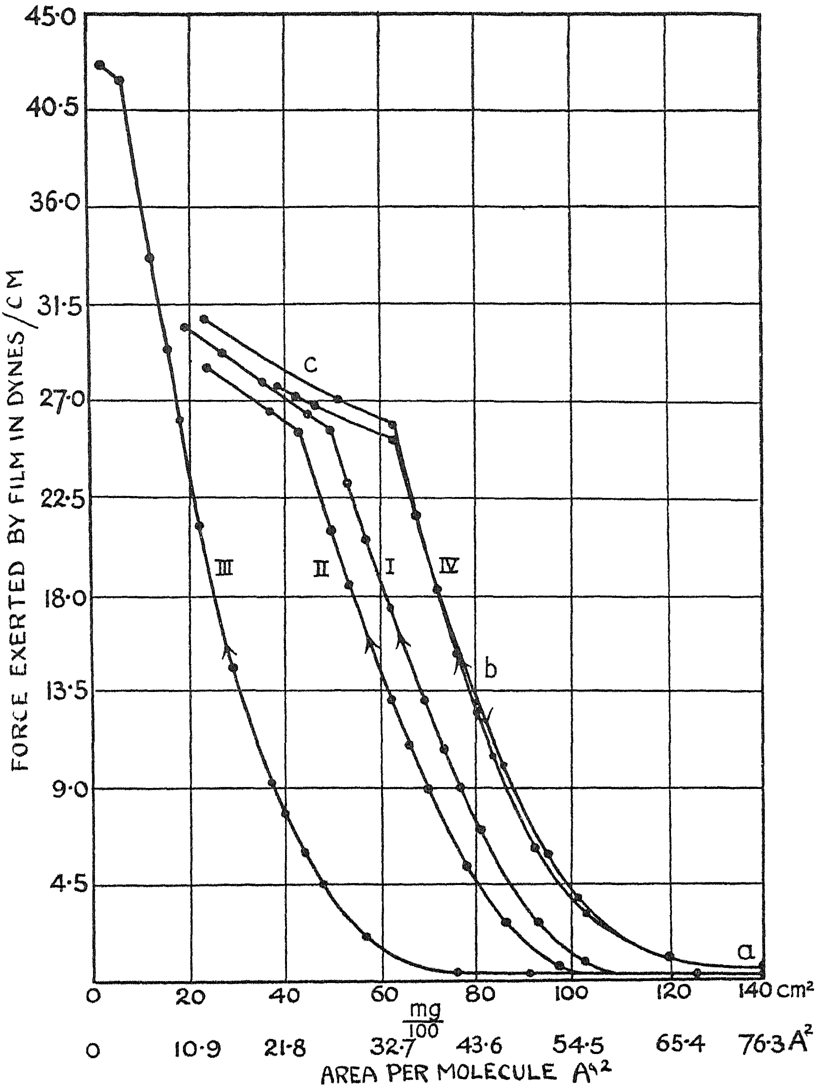

FIG. 1

cule at this point is 34 A², so that the molecules have been packed till they occupy about half their original area. Over this range of pressure (1 to 26 dynes) and area (65 to 34 A²) the film was of the liquid expanded type.

At pressures higher than 26 dynes per cm there was a second break in the curve and the film became more easily compressible (part c, fig. 1). The behaviour at this point resembles the transition from a liquid expanded to a condensed film. Alternatively the fact that part c of the curve is not horizontal may mean that the concentrate is not pure, but contains other substances (perhaps unsaturated compounds) which still continue to exert a pressure. It is interesting that the film remained liquid even at pressures as high as 31 dynes per cm, when the film occupied only a small fraction of its original area. This compressed film could no longer be monomolecular, but must have crumpled or piled up; but it still behaved as a viscous liquid. Adam (1930) has shown that long-chain alcohols up to C_{20} remain liquid at room temperatures when highly compressed.

In a private communication of some unpublished work, Danielli and Adam have described a liquid expanded film of vitamin A which is very similar to one of our slightly oxidized films.

Spectroscopic Examination of Recovered Films

In order to determine whether any change occurred in the vitamin A molecule when it was spread as a film over the water surface, a spectroscopic examination of the recovered film was made by Mr. S. D. D. Morris. Films of unoxidized vitamin A were spread as a monomolecular layer on water using the same method as that employed on the Langmuir trough. After standing for 10 minutes in the air the vitamin was recovered by redissolving the film in ether, and the absorption spectrum in the ultra-violet was examined. The spectrum showed little change and the characteristic band at 3280 A was still present.

Adsorption of Vitamin A

Several workers have shown that vitamin A is adsorbed on solid surfaces. Takahashi (1925) has shown that fuller's earth adsorbs the vitamin from a solution in benzene, producing an intense blue coloration on the surface. The vitamin appears to be destroyed on adsorption so that the process is useless as a method of concentration.

Stephenson (1920) had previously found that charcoal removed carotene from butter-fat, but did not affect its vitamin A activity. Moore in some unpublished work has shown that it is possible to extract vitamin A from certain fractions of cod liver oil by adsorption on charcoal and extraction with carbon

disulphide. All this work was mainly qualitative since no strong concentrates of the vitamin were then available, and other constituents as well as the vitamin were adsorbed from the cod liver oil. More recently Karrer, Walker, Schopf, and Morf (1933) have separated a vitamin A concentrate into two fractions by preferential adsorption on lime.

In the present paper complete adsorption curves are recorded for the strong concentrates which are now available and the effect on adsorption of the nature of the surface and of the solvent has been studied. It has been found possible to recover the vitamin apparently unchanged from the adsorbing surface.

Experimental

In the first experiments the vitamin or carotene solutions were shaken mechanically with the adsorbing material in a series of flasks, through which nitrogen was bubbled in order to prevent oxidation of the solute. The adsorbing materials were silica powder, silica gel, powdered glass, and blood charcoal; the solvents were chloroform and cyclohexane. The vitamin A concentrate (blue value 3000) and the carotene (recrystallized) were supplied by British Drug Houses.

The strengths of the solutions of carotene and of vitamin A were estimated by means of a Lovibond colorimeter, the carotene by its yellow colour in comparison with that of a standard solution, and the vitamin A by the Carr-Price reaction.

Table I—Initial strength of solution (10 cc) = 300 blue units = 0·1 mg/cc.

Amount of charcoal taken, in gm	Final strength of solution in BU	Adsorption of vitamin per gm charcoal in BU	Adsorption in mg per gm
0·5	0	6000	2
0·1	2·5	29750	9·9
0·05	10	58000	19·3
0·03	17·5	102000	34·0
0·01	146	154000	51·3
0·005	175	250000	83·3

Influence of Solvent

Both vitamin A and carotene were readily adsorbed by charcoal from cyclohexane. One gram of Merck's blood charcoal (whose surface area was known approximately from the indigo number) caused complete disappearance of the vitamin in 10 cc of solution containing 0·1 mg/cc of vitamin A. Varying weights of charcoal gave the results shown in Table I.

The decrease in the vitamin content of the solution might have resulted from oxidation during the shaking, *e.g.*, by oxygen in solution or present as an impurity in the nitrogen. If this were so, one would expect the decrease to be independent of the amount of charcoal used. Table I shows clearly that is not so.

That the decrease was not due to oxidation by oxygen adsorbed on the charcoal was proved by outgassing a sample of charcoal, when its adsorptive capacity was found to have greatly increased. For this purpose the charcoal was baked out *in vacuo* for some hours in a glass vessel at 300° C and the vessel sealed off and allowed to cool. It was then opened under the vitamin solution, to exclude air. The amount of vitamin adsorbed by the outgassed charcoal was some 2–4½ times greater depending upon the extent of baking out of the charcoal. Since it can be presumed that the baking out removes at least a part of the oxygen from the charcoal, we should expect a smaller loss than before if the removal of vitamin A had been due to oxidation. Loss by oxidation, if such loss exists, is of secondary importance.

To obtain an adsorption curve, 0·01 gm of charcoal were used with varying concentrations of vitamin A.

In fig. 2, curve I, the amount of vitamin adsorbed is plotted against the concentration of the final solution. This indicates saturation of the charcoal with an adsorption equivalent to 1500 "blue units" of vitamin per 0·01 gm of charcoal. From the indigo number the surface area of the charcoal can be taken as 210 sq metres per gm (Rideal and Wright, 1926). The limiting area of the vitamin A molecule on a water surface was approximately 65 A^2. If we assume that the molecule occupies the same area on a solid surface a monomolecular layer of vitamin A is equivalent to 7100 blue units per 0·010 gm charcoal and the charcoal surface is saturated with the vitamin at approximately 0·2 of a monomloecular layer.

A similar adsorption curve was obtained in chloroform solution and is shown in fig. 2, curve II. The adsorption is much less, and the curve indicates saturation of the surface at approximately 0·05 of a monomolecular layer. It is probable, however, that the molecules would lie flatter on a solid surface and so occupy a larger fraction of the surface.

These results show that vitamin A is more readily adsorbed on charcoal from the non-polar solvent, cyclohexane, than from the polar solvent, chloroform.

THE INFLUENCE OF SURFACE

In fig. 2, curves III, IV, V, and VI, are plotted for the adsorption of vitamin A from cyclohexane solution on surfaces of outgassed charcoal, silica powder,

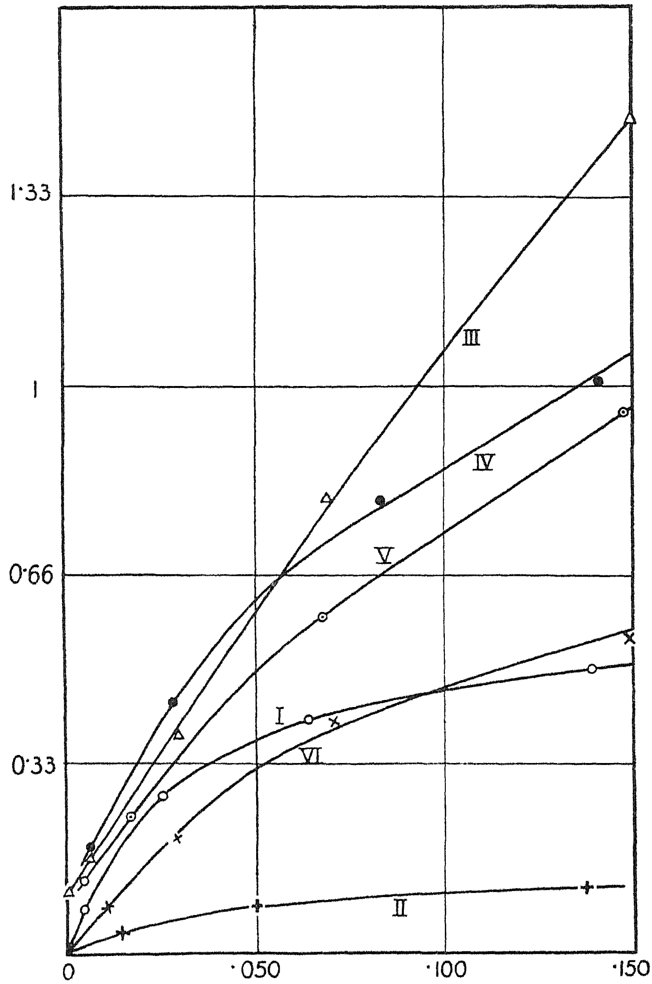

FIG. 2—I, 0·010 gm charcoal in cyclohexane; II, 0·010 gm charcoal in chloroform; III, 0·010 gm outgassed charcoal in cyclohexane; IV, 2·5 gm silica in cyclohexane; V, 1 gm silica gel in cyclohexane; VI, 2·0 gm glass in cyclohexane.

silica gel, and powdered glass respectively. It will be seen that 2·5 gm of silica powder were used, as against 0·010 gm of charcoal, since the charcoal adsorbs a much larger amount of the vitamin than the silica. The surface area of the charcoal is, of course, relatively great, and a truer comparison of the

adsorptive capacity of the respective surfaces can be obtained by calculating the amount of vitamin adsorbed by equal surface areas.

A microscopic examination of the powders indicated that the surface area of the silica powder, taking each particle as a cube, was approximately 5 sq metres per gram, and that of the glass somewhat less, 2 sq metres per gram at a very rough estimate.

The calculated adsorption per square metre of surface is shown in Table II.

Table II

Surface	Maximum adsorption of vitamin A per gram of substance in mg	Adsorption per square metre in mg
Charcoal	50	0·25
Powdered silica	0·53	0·1
Powdered glass	0·41	0·2

It will be seen that the adsorption per square metre in each substance is of the same order indicating that it is the area of the surface rather than the chemical nature of the surface which is the most important factor under these conditions. For the silica gel and the out-gassed charcoal no reliable surface areas could be calculated, though presumably they would be respectively greater than those of the silica powder and the untreated charcoal. Both substances showed a greater adsorption.

Adsorption of Carotene

Curves for carotene* are shown in fig. 3, curves I and II, and may be compared with similar curves (III and IV) for vitamin A. It will be seen that the carotene is adsorbed more strongly than vitamin A from cyclohexane.†

Recovery from Surface

Preliminary experiments had shown that it was possible to recover the adsorbed vitamin from the charcoal by means of a suitable solvent. This recovered vitamin was apparently unchanged and could be estimated by means of the Carr-Price reaction.

* Crystalline β-carotene containing a small amount of α-carotene.

† Since the completion of this work, a paper describing the relative adsorptions of vitamin A, carotene and cholesterol in various solvents on "norrit," silica, and alumina has been published (Holmes, Lava, Delfs, and Cassidy, 'J. Biol. Chem.,' vol. 99, p. 417 (1933)). The authors also find that carotene is more strongly adsorbed than vitamin A.

Further experiments were made to determine the extent to which it could be recovered, and the possibility of a further concentration by this means of the original concentrate.

Vitamin A (87·7 mg) was adsorbed on charcoal from a cyclohexane solution. The charcoal was then filtered off and washed with chloroform, successive washings extracted 15, 12·3, 6, 7, and 3 mg of the vitamin, a total of 49 mg, so that 56% of the adsorbed vitamin was recovered. There was, however,

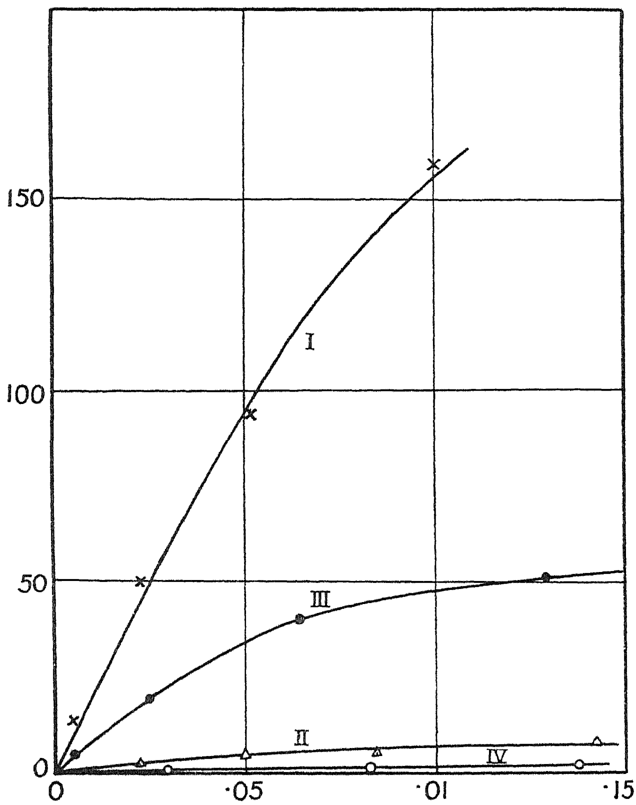

FIG. 3—I, Carotene on charcoal; II, carotene on silica; III, vitamin A on charcoal; IV, vitamin on silica.

definite spectroscopic and colorimetric evidence of oxidation of the vitamin, probably due to exposure of the solution to air while filtering and washing.

Attempt to Concentrate Vitamin A by Adsorption

If the more active fraction of the concentrate were adsorbed more strongly on to a charcoal surface, the vitamin might be concentrated by adsorbing this active fraction on charcoal and extracting with a solvent.

The experiment was carried out with the apparatus shown in fig. 4, designed to minimize loss by oxidation. A strong solution of the vitamin in cyclohexane, together with a quantity of charcoal, was placed in the bulb E, over the porous plug F. A stream of nitrogen bubbling upwards through the plug, acted as a stirrer and prevented the solution from running through. After about 30 minutes the cyclohexane was allowed to drain off, and the charcoal was washed with chloroform. The solution was collected in the bulb H which was then sealed off at G. The chloroform was pumped off and the bulb placed in an electric furnace with the thin arm water cooled. The temperature was

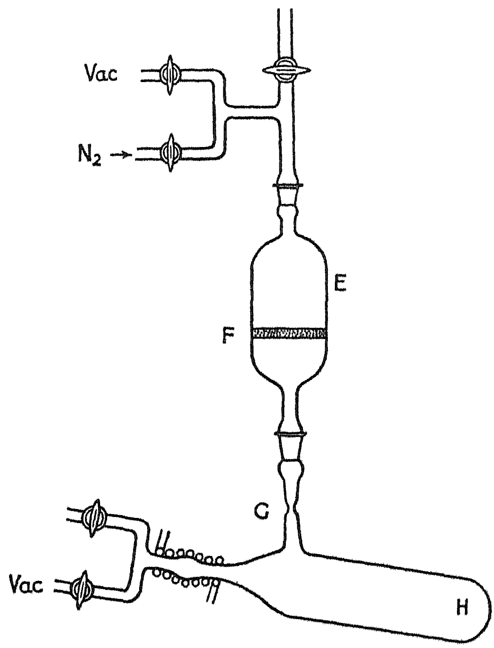

FIG. 4

slowly raised to 170° C and the vitamin distilled over. The bulb was then broken and a weighed amount of this deposit was dissolved in chloroform, and its strength estimated spectroscopically and by the Carr-Price reaction. It was only 82% as strong as the original British Drug Houses concentrate and was found to have oxidized slightly.

Adsorption on Animal Tissue

Some experiments were made to determine the extent to which animal tissue could adsorb vitamin A and carotene. It was found that the pulped liver of

an " A free ' rat could take up vitamin A (1·8 mg per gm) from a dilute cyclohexane solution (0·5 mg per cc) while carotene was not adsorbed at all. It is obviously unsafe to draw any analogy between the behaviour of liver under these conditions and *in vivo*, and these experiments were of a very preliminary nature.

It is with much pleasure that we express our thanks to the Commissioners of the Exhibition of 1851 for a scholarship to one of us (S. H. B.) and to Dr. N. K. Adam, Dr. A. H. Hughes, and Dr. T. Moore for constructive criticism.

Summary

Vitamin A in dilute solution shows no abnormal surface properties. It spreads on water to form an insoluble film of the liquid expanded type and the surface behaviour resembles that of a long chain alcohol. It is slowly oxidized by the air with a decrease in surface area.

It is adsorbed by various solid surfaces, though not so strongly as carotene. The degree of adsorption depends on the nature of the solvent; it is more readily adsorbed from a non-polar solvent, such as cyclohexane, than from a polar solvent, such as chloroform. The adsorbed vitamin can be recovered from the surface apparently unchanged.

The vitamin is also taken up from cyclohexane solution by freshly killed liver tissue whereas carotene is not adsorbed.

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Observations on the Structure of Striated Muscle Fibre

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(Communicated by W. E. Agar, F.R.S.—Received March 29, 1934)

[Plates 1–4]

In this paper one feature only of the structure of striated muscle fibre is considered, namely, the interpretation of the striations.

It is generally held that the striæ of muscle fibres are the expression of a succession of transverse discs viewed in thin optical section. In a short paper in 1922 I described appearances in muscle fibres of insects and of vertebrates which were inconsistent with this interpretation, and which seemed to show that the striæ were the optical expression, not of a series of discs, but of a double spiral membrane (double helicoid) traversing the length of the fibre. The following description is based on a re-examination of a large quantity of new material, mainly of vertebrate origin.

Historical

A fuller acquaintance with the vast literature on muscle structure reveals that the conception of a spiral structure of the fibres, which at the time of publication I regarded as new, has been independently reached by several observers. For some of the following references I am indebted to Professor J. T. Wilson, of Cambridge, and to Professor Boeke and Dr. Eutz, of Utrecht.

In 1715 van Leeuwenhoek, in a letter to the Royal Society of London, described the striations of muscle fibres and offered the opinion that they were in the form of a thread wound spirally round the fibre. In a celebrated paper in 1840 Bowman pointed out a serious error in this conception; for he observed that the striæ could be focussed within the interior of the muscle fibre and that by maceration they could be split into what seemed to him to be discs. Bowman concluded that the fibres were built of a succession of transverse discs, a view now generally accepted.

Fulton in his " Selected Readings in the History of Physiology " brought to light a statement by Rouget (1867) that " the elementary fibril (*i.e.*, fibre) of the striated muscles is, like the style of *Vorticella*, a ribbon twisted into a

spiral whose turns are separated in the lengthening and come together during contraction of the muscle."

In 1895 Davay described a spiral arrangement of the striations of crustacean muscle; his illustrations show not only single but also double and triple spirals. Münch in 1903 independently came to a similar conclusion; he believed he could detect a spiral organization of the striæ of vertebrate muscle, and demonstrated it quite clearly in insect tissue, where it is more readily seen.

Heidenhain in 1911 referred to a peculiar "vernier" appearance of the striations of heart muscle, which he interpreted as a spiral cut in thin longitudinal section. In a later paper (1919) he described fibres in which the complete spiral was actually seen. He regarded the spiral as an uncommon variation of the ordinary disc arrangement.

In 1921 Woerdeman, developing Heidenhain's idea, concluded that in many cases the Z membrane (Krause's membrane) formed a spiral and that the arrangement of the anisotropic part was therefore also spiral. Perhaps the whole muscle fibre might contain one spiral; but in most cases within the fibres were several bundles of myofibrils, each of these exhibiting the cross striation as a spiral. The same seemed to occur in heart muscle, though a definite conclusion was even more difficult to reach here.

D'Ancona (1929, 1930) has examined the matter in specially favourable insect material and is able to confirm these observations in the living muscle fibre; he concludes that the fibres are built on a simple single helicoidal plan.

The Appearance of Helicoids in Longitudinal Optical Section

To observe the striæ of muscle fibres with precision, moderately high power lenses are necessary. Consequently even with binocular arrangements it will not be possible to see at a single focus more than a very thin optical section of the comparatively thick muscle fibre. Since the appearance of a muscle fibre when the plane of focus is taken at different levels within the thickness of the fibre is often very perplexing, it will be useful to consider, as a preliminary, the figures yielded by helicoids in longitudinal section.

Let fig. 1 represent a symmetrical single helicoid, contained in a hollow cylinder. Let BNCMA be a single turn of the helicoid. Now suppose that, starting from the nearer side, the helicoid be cut into successive thin sections parallel to its axis. Unless the section passes exactly along the axis this will yield merely a series of parallel lines (striæ) and the direction of the lines in

sections cut from the nearer half of the helicoid will be at an angle with those in sections from the opposite half, the size of the angle depending on the dimensions of the helicoid. Consider now a thin slice taken along the axis but sufficiently thick to include a contribution from both halves. The section will have a peculiar zig-zag appearance composed of a series of alternating Y-figures as indicated in fig. 1. (The term Y-figure is more convenient than accurate; fig. 1 will show what it is intended to convey. The bent lines BO_3O_1 and AO_2O_1 are the limbs of the Y, CO_1 its stem.) Suppose now a helicoid be examined at successive depths of focus in thin longitudinal optical sections. Immediately below the level M there will appear a series of parallel straight lines. But as the focus passes deeper into the fibre these will assume more and more the form of the line CO_1O_2A. As the focus approaches the axis of the helicoid CO_1O_3B gradually becomes a straight line, so that when the focus is exactly along the axis, both CO_1O_2A and CO_1O_3B are simultaneously visible as continuous lines, and the characteristic Y-figure is seen. As the focus enters the distal half of the helicoid CO_1 separates from O_2A, while CO_1O_3B gradually approaches a straight line.

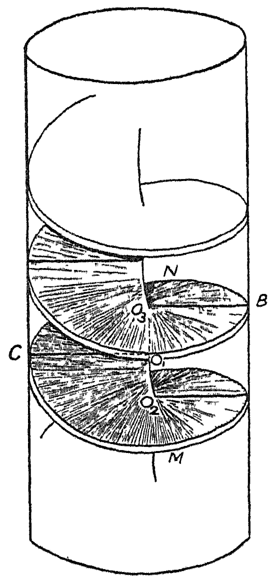

Fig. 1

The lines O_1O_2 and O_1O_3 are of particular interest. On account of the peculiar slope of the helicoid in this region (a solid model will make this clear) O_1O_2 and O_1O_3 may often be only indistinctly visible. Hence in focussing through the axis of the helicoid the effect may be obtained of the line CO_1 abruptly parting from O_2A and passing over to O_3B; for descriptive purposes we may call this the "broken striation" effect.

If the helicoid be tilted so that its axis is slightly inclined to the plane of focus, then if the latter intersects the axis this effect, which can otherwise be obtained only by changing the focus, is now seen gradually developing over a succession of striæ, at a single focus (fig. 2). This is the vernier* effect of Heidenhain. In practice one is not apt to encounter verniers uncontaminated by a trace of Y-figures, because it is unlikely that CO_1, AO_2, and BO_3 can be

* The term "vernier" is, of course, not strictly applicable since the distance between the striæ of the $n + 1$ series is not proportionally smaller than that between those of the n series. The term is retained for convenience.

brought into focus simultaneously, without either O_1O_2 or O_1O_3, or even both being also visible. Contrast in this respect fig. 2 with fig. 10, Plate 1. Clear cut verniers may be expected when the striæ are far apart; for at its axis the

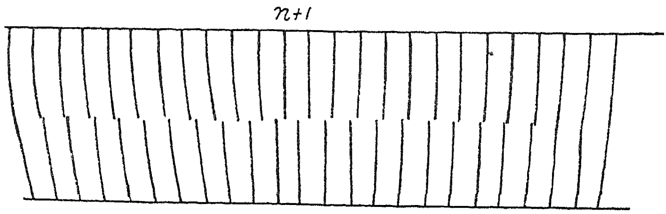

FIG. 2

helicoid must then have a specially sharp turn which would make it hard to see in longitudinal optical sections. The number of striæ involved in the vernier depends, of course, on the angle between the plane of focus and the axis of the helicoid.

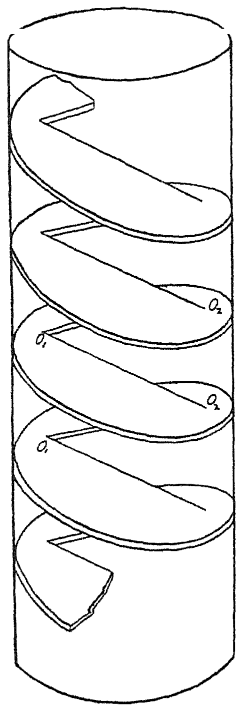

FIG. 3

Consider now a " helicoid " of the kind shown in fig. 3, *i.e.*, a " helicoid " wound about a plane surface as axis and presenting therefore two series of sharp turns, O_1 and O_2. The appearance of the helicoid will depend on the angle from which it is examined.

(*a*) If the levels O_1 and O_2 are simultaneously in the plane of focus as will more easily happen if O_1 and O_2 are close together, then a zig-zag effect must occur of the form shown in fig. 4. If the level of focus be taken very slightly above the plane passing through the points O_1 and O_2, then the striation CO_1aO_2A will be sharply in focus while the line O_1bO_2 is only dimly visible. But when it is lowered so that O_1 and O_2 are simultaneously focussed the complete zig-zag will be visible, of which the portions O_1bO_2 and O_1aO_2 will be likely to appear less distinct than CO_1, BO_2, and AO_2. With the focus ever so slightly below the level O_1O_2, CO_1bO_2B will come sharply into focus O_1aO_2 being only dimly visible. It is evident that the alternating Y-figures in fig. 1 are only a special case of this condition, the plane surface that constitutes the axis of the helicoid becoming a straight line. The opposite extreme will occur when O_1 and O_2 lie at the margin of the helicoid, *i.e.*, alternating

V-figures (not Y-figures) will occur. It is clear that they are likely to be found in very narrow helicoids.

(*b*) If the helicoid is turned at right angles about its axis bringing O_2 under O_1, then focussing downwards the striation will break as level O_1 is attained, and at the level O_2 one part of this striation will join the opposite half of the adjacent striation.

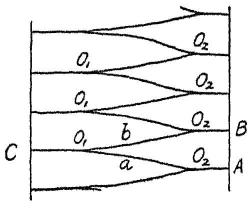

Fig. 4

(*c*) Suppose that the helicoid is examined in a position intermediate between *a* and *b* and that the level O_1 is nearer the observer than O_2. Optical longitudinal sections at different levels will give the following appearances. Sections near the surface will yield a series of parallel lines, striæ, fig. 5, *a*. When level O_1 is reached the striation will appear to branch, fig. 5, *b*, and at a level ever so slightly lower it will divide into two parts, K and L, fig. 5, *c*. As the focus is now gradually lowered K will move towards the next striation, the length of K increasing, that of L decreasing, fig. 5, *d*. Finally at level O_2, K will merge into L, fig. 5, *e*, and below this complete striations will again occur, fig. 5, *f*.

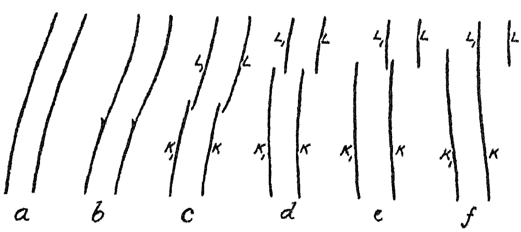

Fig. 5

(*d*) If the helicoid is so placed that the plane of the levels O_1 and O_2 intersects the plane of focus in such a way that the point O_1 of one striation and O_2 of a distant striation are simultaneously in focus, then all the effects which are obtained at successive depths of focus and described under heading (*c*) will be given at a single focus in a vernier effect which passes obliquely across the helicoid from the point O_1 to the particular point O_2 that is simultaneously in focus, fig. 6.

The above discussion may be extended with but little modification to double helicoids. We have here two helicoids which follow one another, the turns of one being half-way between the turns of the other. Surface sections will as usual yield parallel striations. Sections along the axis of the double helicoid are best understood by reference to some solid model. The common laboratory

bottle-brush is a good example of a double helicoid. If the section be so taken as to include a contribution from both halves of the double helicoid, then there must occur the typical Y, or broken striation, appearances, one Y corresponding to each helicoid. A reference to a model will show that the Y's will be opposed and that their limbs will cross. This is shown in fig. 7, where AOBC and A′OB′C′ are the two Y's the thinner lines, BO and B′O, being viewed in a slightly lower plane than the other four thicker lines.

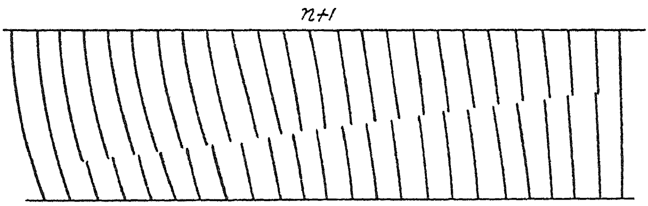

FIG. 6

Suppose such a double helicoid be examined in longitudinal optical section. Sections near the surface will reveal a succession of striæ which at deeper focus will assume the form of the line AOC. Immediately below the axis of the helicoid AOC will become BOC with the production of Y-figures or broken striation effects. At the transition point the line CO will momentarily come opposite OC′, *i.e.*, a Y-figure, namely, C′OAC will form, which will give place to another Y-figure BOC′C at deeper focus. If broken striation effects occur instead of Y-figures, then we should have the appearance of the line CO separating from AO, joining momentarily on to OC′ and with slightly deeper focus becoming continuous with BO. Hence the appearance of simple Y-figures (such as C′OAC) will not be inconsistent with the actual presence of a *double* helicoid.

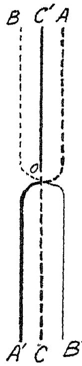

FIG. 7

Optical longitudinal sections along the axis of double helicoids of the type shown in fig. 3 will depend on whether or not both O_1 and O_2 are simultaneously in focus. If both are in focus and if Y-figures occur, the effect will be as in fig. 8. Since, as already stated, the lines O_1O_2 may appear a little blurred, it is not likely that the complete *double* helicoid effect could be seen at one single focus, except in very special material (such as well-stained preparations of thin muscle fibres).

If the level O_2 is out of focus when O_1 is in focus, the lines O_1O_2 will be shortened, and various transitional stages between fig. 8 and fig. 5, *b*, wil

occur. Appearances resembling fig. 4 are special cases of this; hence the appearance of a single zig-zag line will not be incompatible with the actual presence of a double helicoid.

Appearance of the "Striations" of Muscle Fibres when seen in Longitudinal Optical Section

If isolated muscle fibres with sharply defined striations are examined at moderately high magnifications appearances are obtained which are inconsistent with the view that they are optical sections of a succession of discs. The accompanying photographs will serve as familiar examples (figs. 9 to 18, Plates 1-4). At a first glance one is apt to dismiss them as post-mortem distortions. But once some facility has been gained in observing them in prepared tissue, no difficulty is experienced in finding them also in living muscle.

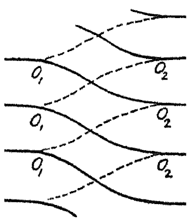

Fig. 8

The possibility that the appearances are some form of optical illusion or artefact will be considered below; but to prevent misunderstanding one source of possible confusion may be discussed here. The depth of focus of an oil immersion lens (2 mm) may be taken at about 0·5 μ. The thickness of ordinary vertebrate muscle fibres ranges from about 30–100 μ. Hence at any particular focus only an exceedingly minute fraction of the total thickness of the fibre can be visible. But if the striations which appear in thin optical sections are in any way bent they are apt to produce the illusion of three-dimensional figures. For instance, in fig. 12, *a*, Plate 2, it is almost impossible to avoid the belief that we are viewing a grooved surface; actually the photograph is in the interior of an isolated intact muscle fibre where such a possibility is excluded. In any case a grooved surface viewed with a microscope in transmitted light would never present the appearance of a groove as seen with binocular vision in reflected light. Similarly in fig. 12, *b*, Plate 2, we have the illusion of a perfect cylinder; actually it is the same fibre as in fig. 12, *a*, taken at deeper focus. The illusion is of the type produced by an engraver who uses the device of giving a particular form to his contour lines to obtain three-dimensional effects on a two-dimensional surface. It is necessary to stress this because the temptation to put a false three-dimensional interpretation on these thin optical sections is often wellnigh irresistible.

Before describing certain special forms of muscle fibres that are particularly favourable for these observations it will be best to consider the ordinary coarse muscle fibres that comprise the skeletal muscle of vertebrates.

Examined at high magnifications the striæ appear as a succession of parallel lines. In the living isolated muscle they may be straight or bent; their actual form is of no significance, for it may alter from time to time in the living fibre in accordance with local conditions of stretching. Such straight or bent lines may be interpreted as optical sections of flat or bent discs. But as the focus is gradually lowered into the thickness of the fibre different appearances arise. One of the commonest is the following.

At a certain level of focus a whole succession of striations will each develop a "kink" so that the two halves of the striation are no longer in perfect alignment. As the focus is gently lowered the "kink" becomes blurred, the remainder of the striation appearing with undiminished clearness. As the lens is further lowered the "kink" passes completely out of focus, one half of the striation becoming separated from its fellow. Focussing still deeper, the one half now gradually joins on to the opposite half of what was at a higher focus, the adjacent striation. The junction appears at first as a blurred "kink" which becomes more definite at a yet lower focus, till finally a perfect striation becomes restored.

For purposes of objective illustration* a common manifestation of this "broken striation" effect may be used. It often happens that the longitudinal axis of the fibre is not perfectly in the plane of focus. The "broken striation" is not then in focus simultaneously for a whole succession of striæ, but for only a limited number; but over this limited range all the optical effects, which otherwise are obtainable only by gradually altering the focus now appear in order at one single focus over a succession of striations (fig. 9, Plate 1); this is the vernier effect of Heidenhain. Note the change in direction of the striæ at the two ends of the "vernier." This does not, of course, constitute a perfect "broken striation" effect, since the blurred kink is never wholly out of focus. Perfect "broken striations" are, in fact, only very seldom seen in typical coarse muscle fibres.

* Although the present description is based on an examination of both living and preserved tissue, the photographs are from the latter only (mainly human material). Preserved material has necessarily been used because from such material alone is it possible to obtain uninjured fibres in a degree of isolation sufficing for convincing objective illustration. The isolated intact fibres have been photographed unstained, mounted in glycerine. For microtome-sectioned material hæmatoxylin staining has been used.

In stained preparations these "vernier" effects may be seen to perfection. An example is given in fig. 10, Plate 1. If any particular "broken striation" in such a vernier period be selected, then by gently lowering or raising the lens a perfect striation is reformed provided, of course, that the section is thick enough; and in the process of reforming such a striation all the phases that appear to the one side or the other of the selected striation now appear in succession as the focus is gradually lowered or raised. At the same time the vernier moves on to a different set of striations.

If in a vernier composed of n and $n + 1$ striæ any selected member of the n series has moved relatively to the right to join one of the $n + 1$ series, then the vernier period as a whole moves to a set of striations to the left; and *vice versa*. These effects are shown in fig. 10, *a*, *b*, and *c*, Plate 1. Three photographs from the same fibre have been taken at slightly different levels of focus. Fig. *b* is at a level intermediate between *a* and *c*. At the particular level of focus selected, the striation numbered 11 in the n series in fig. 10, *b*, is in a position exactly intermediate between 11 and 12 of the $n + 1$ series, and this may be chosen as the mid-point of the vernier. At the slightly lower focus shown in fig. 10, *a*, striation 11 of the n series has become continuous with striation 12 of the $n + 1$ series, while the vernier as a whole has moved slightly to the left, its mid-point, using the above criterion, being at about striation 6 of the n series. When the focus is moved in the opposite direction as in fig. 10, *c*, striation 11 of the n series is continuous with 11 of the $n + 1$ series, while the vernier as a whole moves slightly to the right, its mid-point being at 13. Corresponding striations in the three photographs may be identified by reference to the adjacent nuclei. In fig. 11, *a*, *b*, and *c*, the selected striation is shown at the three selected levels of focus at higher magnification; the lettering employed is the same as that of fig. 1. It is evident that the striæ here possess a helicoidal arrangement of the type shown in fig. 1. The type is to be interpreted as one in which the axis of the helicoid intersects the plane of focus; the slight shift of the vernier on to a neighbouring set of striæ in a direction determined by the depth of focus is a necessary consequence of such an arrangement.

In figs. 12 to 17, Plates 2 and 3, are shown muscle fibres in which the striæ form not a simple symmetrical helicoid but one of the type shown in fig. 3. Fig. 12, *a*–*e*, Plate 2, shows a single muscle fibre at five successive levels of focus; fig. 5 should be used for comparison. At the level of focus shown in *a* the striæ present at one point a slight irregularity or blurriness where the break is about to take place. At the lower level *b* the break has occurred and is

commencing to move across the fibre. At a yet lower level *c* it has moved farther across, and more so at a still lower focus *d*. Finally at the lowest level of focus *e* perfect striæ have reformed almost at the margin of the fibre. Evidently a helicoid of the type shown in fig. 3 is being viewed from a direction discussed under heading (*c*) of the accompanying text.

The photographs do not provide actual objective evidence that the reconstructed striæ at level *e* are composed of two distinct parts, namely, K and L_1 (and not L), using the lettering of fig. 5; although in the actual muscle fibre it is not difficult to observe this as the level of focus *e* is being attained. For objective (photographic) evidence that such reconstruction of striæ really does occur vernier effects must be relied on. Such verniers, as already discussed, must pass obliquely across the fibre. In practice they may be obtained in two ways: (*a*) in microtome sections, where some of the fibres may accidentally be cut in the appropriate plane. Examples of oblique verniers are readily found in such tissue (fig. 14, Plate 3); (*b*) a part of the fibre is raised by laying it across a very fine glass capillary; on the sloping portion of such a fibre the oblique vernier then becomes visible at a single focus.

Fig. 13, Plate 3, is a photograph of a fibre prepared in this way. That the plane of focus does, in fact, intersect the longitudinal axis of the fibre is shown by the right end of the fibre being partially out of the plane of focus. The vernier is seen running obliquely across the fibre. This photograph is the best that I have so far been able to secure of the oblique vernier. Although the striæ at the left end of the fibre are not quite complete, yet it is possible to see in succession along the vernier the various phases shown at different levels of focus in fig. 12, Plate 2 (compare heading (*d*) accompanying description of fig. 3).

Figs. 15–17, Plate 3, show some of the appearances commonly presented by this type of fibre. In fig. 15 the optical section of a helicoid may be seen to perfection; note that the dark striations on one side of the fibre alternate with those of the other half, so that the dark line travels in a zig-zag along the length of the fibre (*cf.* fig. 4). Although the zig-zag will probably be obvious at a glance, it is possible to put a different though false interpretation on this illustration. The dark bands of the one side in alternating with those of the other necessarily come into alignment with the pale bands of that side. This easily leads to the false impression that the dark lines merely become paler as they pass across the fibre; under such conditions the zig-zag may not even be seen. This interpretation is, of course, wrong, as may readily be proved by identifying the pale line by the presence of Dobie's line (D.L.) within it. To

avoid confusion several of the dark striæ of opposite halves of the fibre are indicated by lettering (*s*).

While the foregoing observations show that at any rate in the particular fibres examined a helicoidal arrangement of the striæ occurs, they do not, for reasons already discussed, reveal whether the helicoid is single, as D'Ancona (1929) believes, or double, etc. It is evident that the matter can be decided only in whole fibres, not in sections, where portion of the fibre is apt to be cut away by the microtome knife. In isolated whole fibres, from material in which the striæ are exceptionally clear, I find again and again that when the fibre is so focussed as to bring into view the " broken striation " effect, then by gradually lowering the focus, one half of the " broken striation " can be followed past the opposite half of the adjacent striation which it appears only momentarily to join, to become continuous with a striation one in advance, *i.e.*, in these cases the helicoid is double not single. Whether the helicoid is always double in these fibres is extraordinarily difficult to determine. In many specimens I have not been able to see it with certainty, but precise observation is often so difficult, that to exclude it on such grounds alone would be unsatisfactory.

More exact observation is possible with certain very thin fibres present in the extrinsic eye muscles. In the rabbit, which has been used in the present work these may range from 4 μ to 10 μ in thickness. Thick microtome sections have been used. In many of the fibres a double helicoid can be detected without difficulty (fig. 18, Plate 4). But there occur other fibres where the helicoid is only single, and it is impossible to explain them away on the ground that a portion of the fibre has been cut away with the microtome knife. Such observations suggest that in coarse fibres single helicoids also may occur, as D'Ancona believes (1930).

These very narrow fibres, whose thickness sometimes scarcely exceeds the distance between successive striæ, deserve special attention ; for the possibility of optical illusion which one often suspects for the coarse fibres does not occur here. The different forms of helicoid whose existence has been detected in the coarse fibres are also recognizable here. Thus in fig. 20 a simple single helicoid approaching in type that shown in fig. 1 is present, while figs. 19 and 21, Plate 4, are obviously optical expressions of helicoids of the type shown in fig. 3.

Striking examples of double helicoids are often encountered in this material. Usually much adjustment of depth of focus is needed to display them in their entirety. An exception is shown in fig. 18, from a fibre 8 μ in diameter. The camera lucida drawing has been made with much adjustment of depth of

focus; it should be explained that when in the drawing the helicoid (zig-zag) becomes incomplete, this is due to part of the fibre having been cut away with the microtome knife. The photograph of this fibre shown in fig. 18, *a*, will serve as more objective evidence. It is, of course, impossible to view more than a single plane of focus in the photograph; despite this the disposition of the striæ in a complete double zig-zag is visible in the region between the asterisks. In these very narrow fibres striæ showing V- rather than Y-figures predominate. The reason for this has already been explained.

The Possibility of Artefact

This is a matter which requires the most careful attention. At least two sources of error are concerned: (1) optical illusion, (2) distortion.

(1) *Optical Illusion*—In focussing through thick colourless muscle fibres the possibility is continually forced on one's attention that the appearances which have been interpreted as originating in helicoidal structures are merely some peculiar form of optical effect due to reflection of light from the sides of the discs. The obvious reply is that the effects can be seen even more clearly in microtome sections (fig. 10, Plate 1) and in the very narrow fibres of eye muscles in which the thickness of the fibre may sometimes scarcely exceed the distance between the striæ (fig. 21, Plate 4).

(2) *Distortion*—Muscle fibres are notoriously prone to distortion. Severely distorted fibres are, of course, never a source of error. The possibility that a less extensive distortion is the source of the effects here described is answered by the fact that once one has become accustomed to observing these effects in fixed preparations one has no difficulty in seeing them also in living muscle. The parallel fibred thin sartorius of the frog is a useful muscle to employ, for isolation of individual fibres is there unnecessary. To allay the suspicion that even in this case the effects may be due to distortion of the living fibres when their bony attachments are severed, it may be remarked that the same effects can be observed in living muscle fibres of suitable arthropods when viewed through the transparent leg cuticle. My own observations have been made on the transparent legs of a spider. By focussing immediately under the cuticle it is usually possible to observe several fibres in complete isolation from their neighbours. They are normal living fibres, for they often exhibit contraction waves. In these fibres the spiral organization of the striæ is often quite clearly visible. Fig. 22, *a*, *b*, and *c*, Plate 4, shows three drawings of such a living

fibre, at three levels of focus; the zig-zag is at intermediate focus and is evidently along the axis of the helicoid.

Reference may be made here to two criticisms which have been raised against these views. According to Woollard (1930) the effect "is due to the dark appearance of the longitudinal bands. These shift in position as the focus is varied and this is the explanation of the apparent continuity of Krause's membranes." It does not seem to me that this criticism has any real bearing on the observations here recorded. A recent criticism by Jordan (1933) that the helicoid is "the result of shearing stresses or oblique tensions" is answered by the fact that it can be seen in the living untouched muscle fibre; and a further criticism by the same author "that a spiral structure stretching from end to end of the fibre in the form of a helicoid is disproved by the simple fact that the macerated fibre splits into regular circular discs" would be a valid argument only if it could be shown that they were, in fact, discs and not fragments of the helicoid. As we have seen it is often a very difficult observation to make even in the intact fibre; in macerated fibres it would be impossible.

To prevent misunderstanding it should perhaps be added that fibres with helicoidal striations must not be confused with fibres within which, according to Ballowitz, Marceau, and others, the myofibrils run a spiral course. These are said to occur particularly in molluscs, but according to Plenk (1924) are merely artefacts. In the fibres dealt with in the present paper the myofibrils never run any way but parallel to the long axis of the fibre.

General Remarks on the Occurrence of Helicoidal Striations

Is the helicoidal structure of general occurrence in striated muscle? For highly developed vertebrate muscle which interests us primarily, this is in my experience a most difficult matter to determine. It would be easy on the basis of hasty observation to deny its occurrence in many fibres that one examines; but again and again, in my experience, in fibres where it first failed to appear more careful scrutiny has revealed its occurrence.

It is hardly necessary to say that isolated whole muscle fibres are needed in examining this matter, for in microtome sections portion of the helicoid is apt to be cut away. Obviously distorted fibres as well as fibres in which the striæ are indistinct should also be avoided. In my experience, based on the examination of very large numbers of fibres from human, lizard, guinea pig, and frog limb muscle, it is always present. Probably it always occurs in

human tongue muscle and in the extrinsic eye muscles, where some of the fibres occasionally do not exceed 4 μ in thickness (rabbit material was used). It can readily be seen in most of the narrow fibres from muscle spindles (lizard). It can be seen very commonly in heart muscle, where indeed Heidenhain has already described it; but the question of its constant occurrence here must at present remain undecided, since the striæ are often so faint as to make reliable observation difficult. On the other hand, in the sphincter iridis of the pigeon (where the fibres are thin and striated) a helicoidal structure is only occasionally present. The various forms of muscle fibres of invertebrates have not been systematically examined. In the large somatic muscles of insects its presence can often readily be detected; in the slender striated fibres of the gut musculature it is absent. Invertebrate muscle in general requires further investigation.

From an examination of numerous muscle fibres it has been possible to define at least two common sources of difficulty in detecting the helicoid:

(*a*) The pale line between successive striæ approximately equals the dark band in width. Hence when a " broken striation " is focussed the dark band in one half of the fibre will be in alignment with the pale line in the opposite half. This may give the illusion of a continuous line running across the fibre. This has already been referred to in the description of fig. 15, Plate 3. It is even better seen in fig. 17, Plate 3, where only close scrutiny of the individual bands reveals the broken striation effect. Fig. 23, Plate 4, which is from an exceptionally thick fibre, has been selected to show how elusive this effect may occasionally be. Only close scrutiny of the individual striæ will reveal it in this fibre. The part referred to is the rather pale zone that traverses the fibre obliquely. The lower part of the fibre is, of course, out of focus for the plane of focus has not included the whole width of the fibre.

The fibres here illustrated were selected for photography owing to the sharpness of their striæ; when the striæ are less clearly defined this effect may often be a most serious source of difficulty.

(*b*) Often in focussing into the depth of perfectly intact isolated fibres, at a certain depth of focus vision may suddenly become a little blurred. This is the level at which the break in the striation occurs. Above and below that level vision is perfectly clear. The effect is shown in some of the accompanying photographs (*e.g.*, figs. 16 and 17, Plate 3), but is often much more marked. It appears to have its origin in the fact that, if the focus be taken along the axis of the helicoid, enough of the helicoid (striation) may not be included in the depth of focus of the lens to render sharp vision possible.

When these two features are encountered in one and the same fibre, and particularly if that fibre be very thick, the difficulty of arriving at an interpretation of its structure may be almost unbelievably great.

It is desirable to record these observations since they are a continual source of trouble in detecting the helicoid when large numbers of fibres are under review.

Significance of the Helicoid

This must remain for the time a matter of speculation. That it is not essential for contraction is shown by the fact that muscle fibres exist (insect visceral, and doubtless other invertebrate muscles) in which it does not occur. On the other hand, its apparently constant occurrence in the more specialized muscle of vertebrates, with their power of rapid contraction, requires explanation.

It is a remarkable fact that though the myofibrils are the contractile structures of the muscle fibre, yet the contractile units of these fibrils—the sarcomeres—are disposed side by side in adjacent fibrils with such marvellous precision that they produce the appearance of transverse striæ in the complete fibre. What is the significance of this? If, as now appears, the striæ form a helicoid and not a succession of separate discs, it is conceivable that this helicoid may be the path of the excitatory impulse within the muscle fibre. There has long been known a membrane, the membrane of Krause, between the dark bands and traversing the interfibrillar spaces; it forms in short a *continuous* helicoidal membrane traversing the fibre from end to end. This membrane might well be the portion of the helicoid which is thrown into excitation; it would be the polarizable membrane postulated by physiologists. Such a conception would render intelligible the construction of a muscle fibre, for it would bring the excitatory wave into close association with each sarcomere of the muscle fibre. But beyond the fact that it can be shown in very favourable preparations that the motor nerve ending is connected by fine filaments with the membrane of Krause (Tiegs, 1932) there does not at present exist any certain evidence to support such a hypothesis. Instantaneous photographs of slow contractile waves in insect muscle might throw light on the path of the contractile wave, but they would have to exceed in delicacy anything that has so far been achieved in this field.

In conclusion, it is a pleasure to acknowledge my indebtedness to Dr. E. S. J. King, who has taken all the photographs that illustrate this paper.

Summary

In the large muscle fibres of vertebrates and of arthropods the striæ are the optical expression, not of a succession of transverse discs, but of a helicoid. The helicoid may be double or single. Various forms of "helicoidal" arrangement occur. Forms of muscle fibre exist, particularly in invertebrates, where the helicoidal arrangement does not occur.

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DESCRIPTION OF PLATES

PLATE 1

FIG. 9—Optical longitudinal section of intact isolated human muscle fibre showing a simple "vernier" effect.

FIG. 10, *a*, *b*, and *c*—A muscle fibre, cut in microtome section to a thickness of 5 μ, is shown at three levels of focus, of which *b* is intermediate between *a* and *c*. Corresponding striæ are identified by reference to adjacent nuclei and are numbered accordingly.

FIG. 11, *a*, *b*, and *c*—Portion of the same enlarged. For lettering in fig. *b* compare fig. 1.

PLATE 2

FIG. 12—Five photographs at successive depths of focus of a single muscle fibre. For the geometrical interpretation compare fig. 5, and accompanying text.

PLATE 3

FIG. 13—Oblique "vernier" from a fibre in which the plane of focus intersects the longitudinal axis of the fibre. Refer to accompanying text for description. The effect has been obtained by raising one end of the fibre. Note that the other end is therefore partly out of focus. Part of another fibre is present.

FIG. 14—Oblique vernier from a fibre cut in thin microtome section.

FIG. 15—Longitudinal optical section of a fibre focussed exactly along axis of helicoid. Compare fig. 4 for interpretation. *s.*, striation. *D.L.*, Dobie's line.

FIG. 16—The same.

FIG. 17—The same. This is an example where close scrutiny of the individual striæ is necessary to reveal the broken striation effect.

PLATE 4

FIG. 18—Thin muscle fibre (8 μ) of superior oblique muscle (rabbit), showing double helicoidal arrangement of the striæ. Camera lucida drawing.

FIG. 18, *a*—Photograph of same; the photograph can show only as much of the double helicoid as is visible in a single optical section. The *double* zig-zag is visible in the region between the asterisks. In fig. 18, on the other hand, the drawing has been made with considerable adjustment of focus.

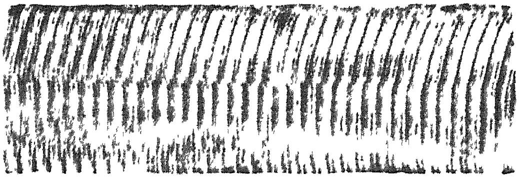
FIG. 19—Thin muscle fibre (7 μ); superior oblique muscle of rabbit, showing single helicoidal arrangement of striæ.

FIG. 20—The same, showing a helicoid approaching the type shown in fig. 1.

FIG. 21—An example of a narrow fibre (4 μ), showing single helicoidal arrangement of striæ. Superior oblique muscle of rabbit. Camera lucida drawing.

FIG. 22—Camera lucida drawing of living muscle fibre of spider, viewed through transparent leg cuticle. Fig. *a* drawn at a level of focus intermediate between *b* and *c*.

FIG. 23—Longitudinal optical section of intact thick human muscle fibre, focussed along the axis of the helicoid. The lower part of the fibre is out of focus. This will serve as an example of a fibre in which only the closest scrutiny will reveal the break in the striation.



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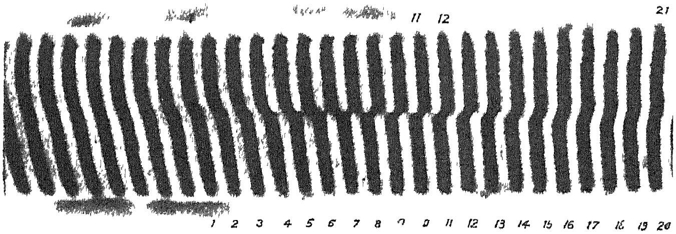

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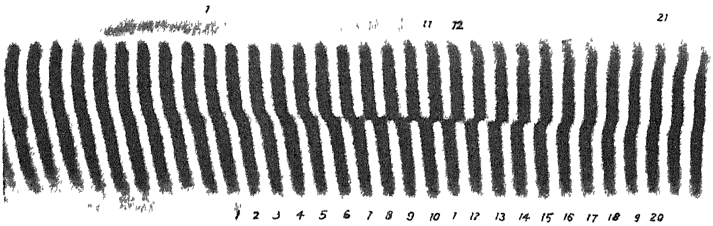

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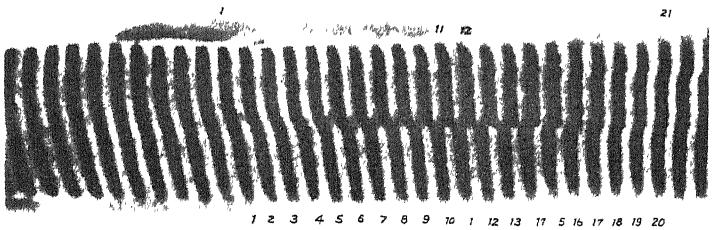

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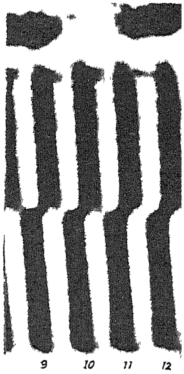

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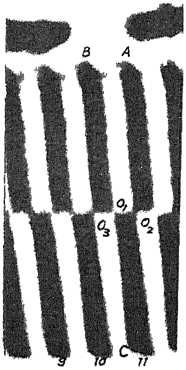

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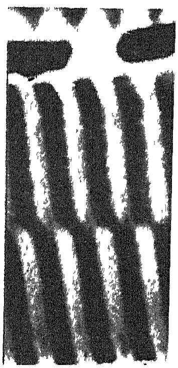

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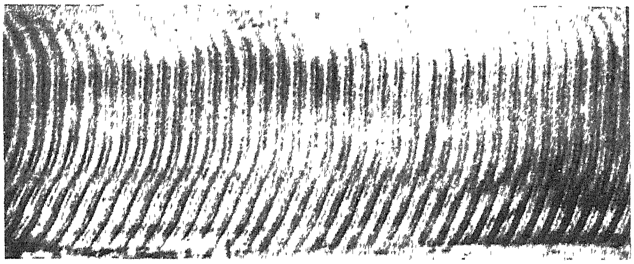
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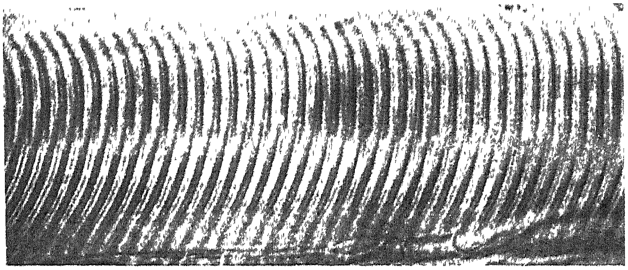
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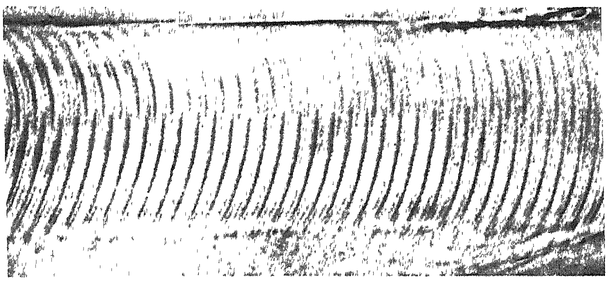
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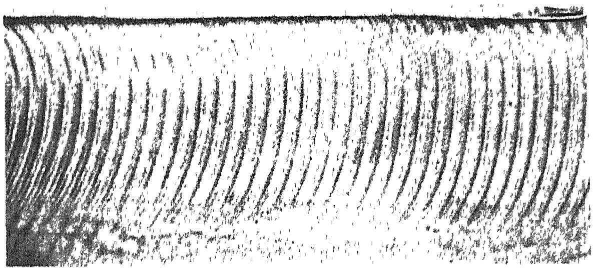
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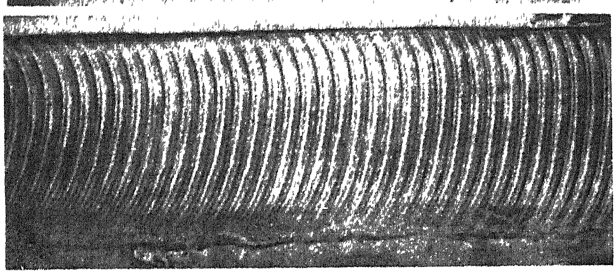
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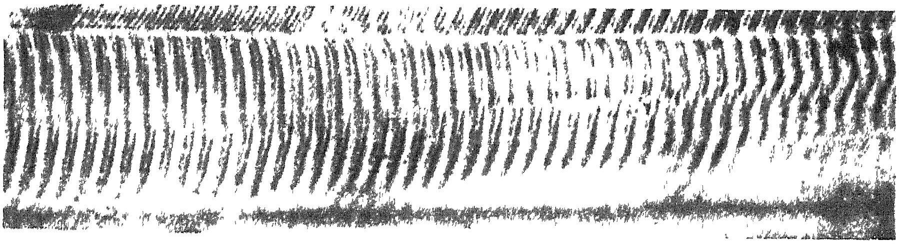

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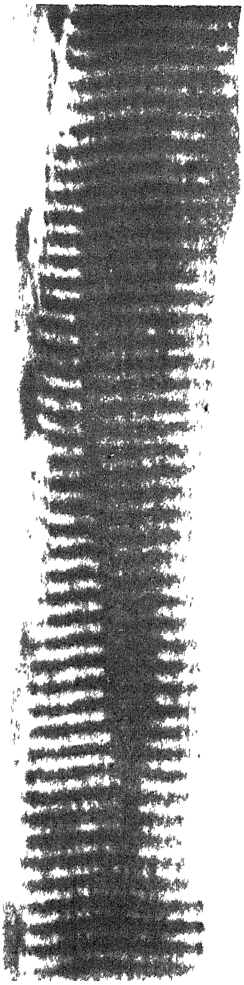

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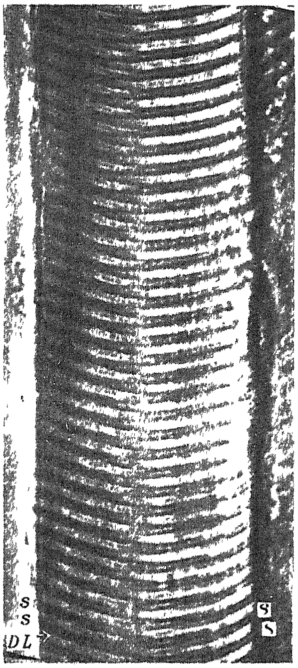

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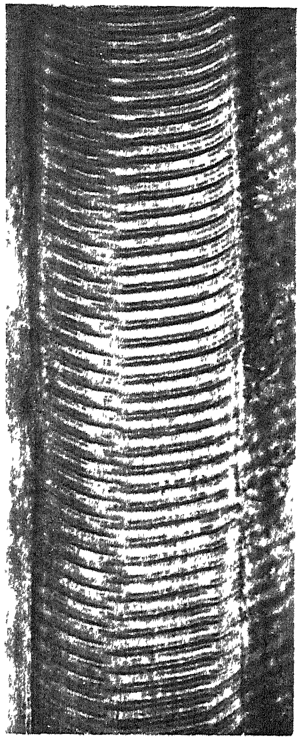

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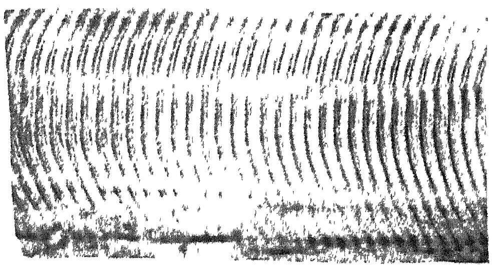

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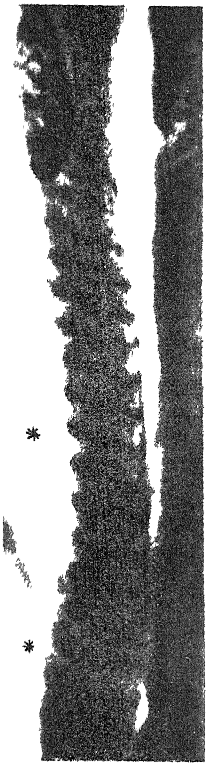

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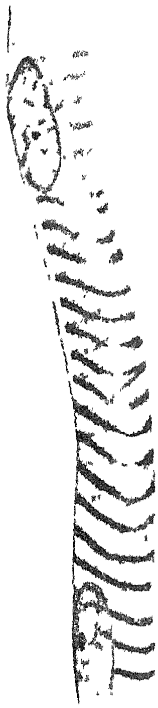

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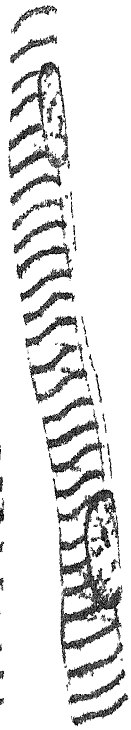

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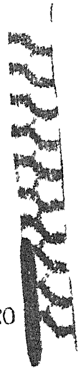

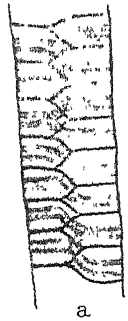

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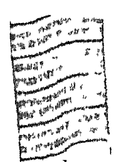

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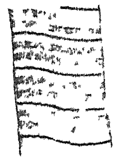

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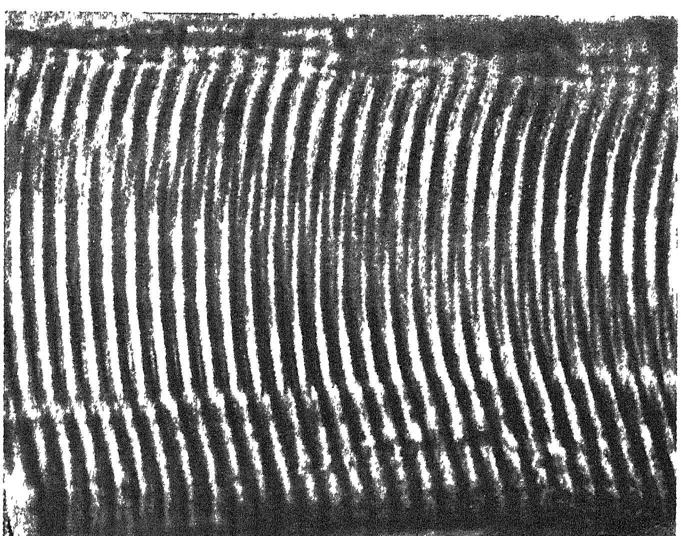

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The Liminal Brightness Increment for White Light for Different Conditions of the Foveal and Parafoveal Retina

By W. S. Stiles, Ph.D., and B. H. Crawford, M.Sc., A.Inst.P.

(Communicated by Sir John Parsons, F.R.S.—Received May 7, 1934)

1—*Introduction*

We have given in a previous paper (1933)* the results of measurements of the liminal brightness increment (l.b.i.) for monochromatic light of different wave-length throughout the spectrum, for various conditions of adaptation of the eye, and for foveal and 5°-parafoveal vision. In the present paper measurements of the l.b.i. will be reported, in which the additional stimulation representing the test spot whose presence or absence is adjudged by the subject, consists of white light or more precisely of radiation with a continuous spectrum corresponding to that of a black body at approximately 2800° K. Conditioning stimulations of the following types have been studied (*a*) uniform background brightness (white), (*b*) uniform surround brightness (white or coloured) with a dark centre field, (*c*) an isolated bright patch or glare source (white or coloured). The test spot was observed either by direct (foveal) or slightly indirect (5°-parafoveal) vision.

In Paper 1, attention was directed to the effect of wave-length on the value of the l.b.i., expressed in energy units, when the eye was subjected to one of a few widely different conditioning stimulations. In the present work, the test stimulus colour is fixed and the interest shifts to the effect on the l.b.i. of progressive changes in the conditioning stimulation, either in regard to brightness or colour. White backgrounds and surrounds of brightnesses ranging from zero to 500 c./sq. ft. and white glare sources producing an eye illumination up to 50 ft. c. have been used. In addition the effect of colour of surround or glare source has been studied. Although the colour of the test spot is fixed, its size and flash period have been varied and the data obtained throw light on the operation of these factors under different conditioning stimulations.

* (Referred to here as Paper 1.) This paper should be consulted for explanations of the terms "liminal brightness increment," "conditioning stimulation," and "test stimulus." as used here.

In Section 3 brief descriptions are given of several minor experiments, which were undertaken to give guidance in the main measurements, but which seem to have an intrinsic interest.

2—*Description of Apparatus*

The apparatus (threshold apparatus I) used for most of the measurements employing white light conditioning stimulation is shown in perspective sketch in fig. 1. The apparatus consists of a large wooden cube of 6 feet side painted white inside. The back wall S is illuminated to any desired brightness level

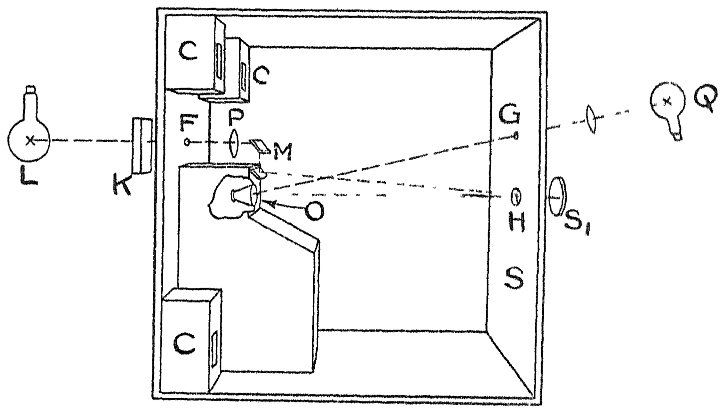

FIG. 1

by four lamp boxes C, in the windows of which diaphragms and filters can be placed. The subject sits in a wooden cabin constructed inside the cube and views the screen S through a suitable aperture O in the front of the cabin. To ensure comfortable conditions of observation for the subject, the cabin is heated and ventilated by a stream of air driven over a heating coil and then through the cabin by a fan. The back wall S contains a large removable panel which can be replaced by other similar panels having suitable holes cut in them to enable a glare source, or a centre field of different brightness, to be exposed to the subject's view. In fig. 1, the panel shown is adapted to expose the image of a pointolite at G, and a small dark centre field at H. The small screen S_1 which receives no light from the lamp boxes C_1 is a white diffusing surface on which the image of the test spot is projected.

The test spot is formed by projecting on to the screen S_1 (or on to the screen S when the centre field is absent) with the aid of a lens P an image of an illuminated aperture in a diaphragm F, the light being obtained from a lamp L

mounted in a carriage which runs on a photometer bench. The brightness u of the test spot is proportional to the illumination in the plane of the diaphragm F. If x is the distance from the filaments of the lamp L to the diaphragm F, we may write

$$u = \frac{K}{x^2}, \tag{1}$$

where K is a constant. In order to obtain a steady rate of change of the test spot brightness arrangements were made to drive the lamp carriage with a motor. It is easy to show that, if the carriage moves at constant speed, the rate of change of the test spot brightness will vary inversely as the cube of x. It was considered that the most suitable rate of change of the test spot brightness would be such that the latter increased by a given proportion of its value in unit time, *i.e.*, such that $\frac{1}{u}\frac{du}{dt} = \text{const.}$ Differentiating equation (1) and dividing by u we obtain,

$$\frac{1}{u}\frac{du}{dt} = -\frac{2}{x}\frac{dx}{dt}.$$

Thus if $\frac{1}{u}\frac{du}{dt}$ is to be constant, dx/dt must be proportional to x.

To contrive a variation in dx/dt of this kind, use was made of the fact that, if a shunt wound motor is run with a constant voltage applied to the field, and a variable voltage applied to the armature, by means of a potentiometer, then the motor speed S varies approximately linearly with the resistance R tapped off on the potentiometer, in accordance with the law:

$$\begin{aligned} S &= a\,(R - R_0) \qquad && \text{for } R \geqslant R_0 \\ S &= 0 && \text{for } R < R_0, \end{aligned}$$

where a and R_0 are constants.

The lamp carriage was therefore driven by a shunt wound motor with a constant 100 volts across the field and with a variable voltage applied to the armature by a contact attached to the carriage and connecting with a potentiometer mounted parallel to and running the whole length of the carriage track. Let $x = x_0$ when the carriage is at the end of its track nearest to F. Suppose the potentiometer resistance per unit length is ρ. The carriage speed at a point x will be proportional to the motor speed and we shall have

$$dx/dt \text{ is proportional to } \rho\,(x - x_0) - R_0.$$

by inserting an additional resistance $R_1 = \rho x_0 + R_0$ in series with the potentiometer resistance on the bench we obtain

$$dx/dt \text{ is proportional to } \rho(x - x_0) + R_1 - R_0,$$

r

$$dx/dt \text{ is proportional to } \rho x,$$

which is precisely the law of variation required.

It was also desired to try different absolute rates of change of u and this was accomplished by using different diameter pulleys in coupling the motor to the chain drive of the carriage.

Four principal carriage speeds have been worked with, corresponding to the following values of $\frac{1}{u}\frac{du}{dt}$: 0·024, 0·061, 0·138, and 0·191 sec^{-1}.

The above values were computed from the data used in the design of the system, and are in satisfactory agreement with direct measurements of the carriage speed in the completed apparatus.

To avoid the necessity of stopping the carriage to record its position when the subject signalled, an electrical recording apparatus was mounted on the carriage, and operated by a Morse tapping key controlled by the subject in the cabin. This recording apparatus marked a black or red dot on a paper tape laid along the bench.

The largest lamps used in the lamp boxes C were 500 watt gas-filled lamps and with these a brightness of the screen S equal to approximately 20 candles/sq. ft. was obtainable.

When the investigation was extended to include coloured surrounds, backgrounds, and glare sources, the maximum intensities obtainable with the above apparatus were found to be insufficient, owing to the necessarily low transmissions of the filters used to obtain light confined to a limited region of the spectrum. This difficulty was met by constructing a smaller apparatus (threshold apparatus II), employing larger lamps. The general plan of apparatus II is shown in fig. 2. Except for the method of producing the test spot, apparatus II is identical with the apparatus described in Paper I and reference should be made to the description there given. For the present work, the test spot was formed as in apparatus I by projecting an image of an illuminated aperture in the diaphragm F on to the small centre field screen S_1, when working with a surround, or on to the continuous back ground screen, when working with a background. The light from the test spot aperture was reflected by a stainless steel mirror M_1 and then by a right angle prism M_2 placed just above the observa-

tion aperture. The lamp illuminating the aperture in the diaphragm F was mounted on a carriage which moved on the bench T. The lamp carriage was motor driven and by a modification of the device already described for apparatus I, the speed was varied in such a way that $\frac{1}{u}\frac{du}{dt}$ remained constant. As before, observations were recorded on a paper tape, placed along the bench, on which a marking device attached to the carriage and operated by the subject impressed a black or red dot.

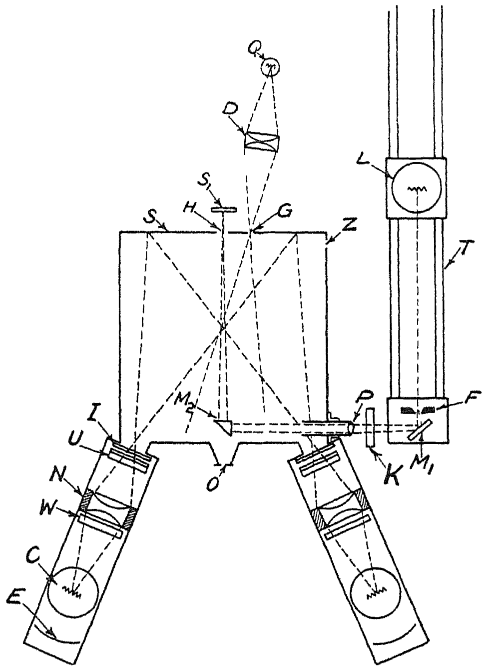

FIG. 2

In both apparatus I and apparatus II, any desired shape and size of the test spot as seen by the subject could be obtained by a suitable choice of the aperture in the diaphragm F. In several series of observations with apparatus II, arrangements were made to rotate slowly in its own plane the diaphragm F. This procedure gave a rotating test spot (of non-circular shape) as seen by the subject.

Instead of exposing the test spot continuously to the view of the subject in much of the work a flashing test spot was used. For exposures greater than about $0 \cdot 3$ sec a simple electromagnetic shutter operated from a rotating drum carrying contact segments proved satisfactory. The shutter used was

made from electric bell parts. The rotating drum was made of ebonite with thin sheet brass segments clamped on, and was driven at constant speed by a small electric gramophone motor.

For exposures between 0·3 sec and 0·02 sec a combination of an electromagnetic shutter and a sector disc running at constant speed was used. The electromagnetic shutter is for the purpose of isolating one exposure by the sector disc. The arrangement is shown diagrammatically in fig. 3. When the contact strip on J passes under K one coil of each electromagnet B, C is energized, but the current is adjusted so that it does not actuate the shutters; it can only hold them in the top position. The shutters only work when the metal sector D passes under F or G, energizing the remaining coil of B or C. Coil C cannot

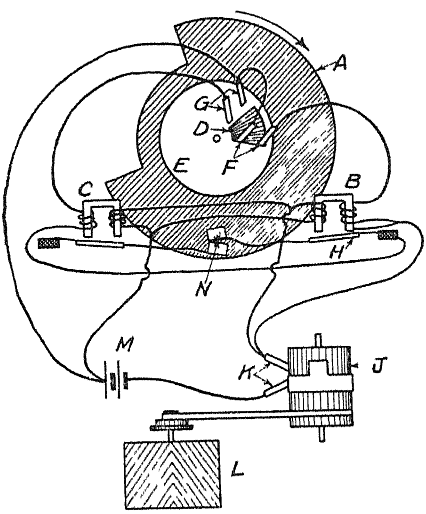

Fig. 3

be energized, however, until shutter B has opened, closing contact H which is in series with coil C. The speed of rotation of drum J must be less than that at which the contact strip passes under the brushes K in a time equal to twice the time of revolution of the sector disc; otherwise the shutter may not always function correctly, since there is no definite synchronization between drum and sector disc. The cycle of operations in each exposure is as follows: contact is made at K, contact is made at F (a possible contact at G has no effect at this stage), shutter B opens, opening in sector passes across aperture N, contact is made at G, shutter C closes, contact is broken at K, shutters drop to first position. It may be noted that the shutter is "self-capping" if B falls before C; this can be ensured by making the spring of the armature of

B slightly stronger than that of C. The speed of drum J regulates the frequency with which exposures are made by the shutter, the exposure time being fixed by the width of the opening in the sector and the sector speed.

The shutter, whichever kind was employed, was inserted in the test spot beam as near as possible to the test spot aperture, *e.g.*, at K in fig. 1.

The light forming the test spot was always white and of relative energy distribution corresponding approximately to 2800° K colour temperature. With the lamp L (either apparatus) in a fixed known position on the bench, a direct measurement of the test spot brightness in candles per square foot could be made by sighting on the test spot an illuminometer, placed at the subject's viewing aperture, all light sources save the lamp L being extinguished for this measurement. A test spot sufficiently large for this to be done was obtained by suitable choice of the aperture in diaphragm F. The test spot brightness for any other position of the lamp L could be at once obtained from the inverse square law (equation (1)). Since very low test spot brightnesses were required, it was frequently necessary to insert neutral filters (whose transmissions were measured) in the test spot beam.

The value of the test spot brightness at which the test spot is on the threshold of visibility represents the liminal brightness increment. We shall use the symbol T for the l.b.i. expressed in the photometric unit—candles per square foot, reserving the symbol U for values where the l.b.i. is expressed in energy units, as in Paper I.

The specification and measurement of the various conditioning stimulations studied, followed closely the description given in Paper I and need not be repeated.

The colour filters employed were the following :—

Filter A′	(blue)	Filter E	(pale red)
Filter C	(green)	Filter F	(red)

The relative energy distributions given by these filters when inserted in a beam of white light from a tungsten lamp of colour temperature of approximately 2800° K are shown in fig. 4, together with the curve for white.

Many of the test spot and conditioning stimulation brightnesses occurring in this paper have very low values, and sometimes coloured conditioning stimulations have been employed. The question may be raised as to the validity of using photometric units to represent such brightnesses. All the low brightnesses here used were obtained from high brightnesses by application of the inverse square law or by interposing diaphragms or neutral filters of

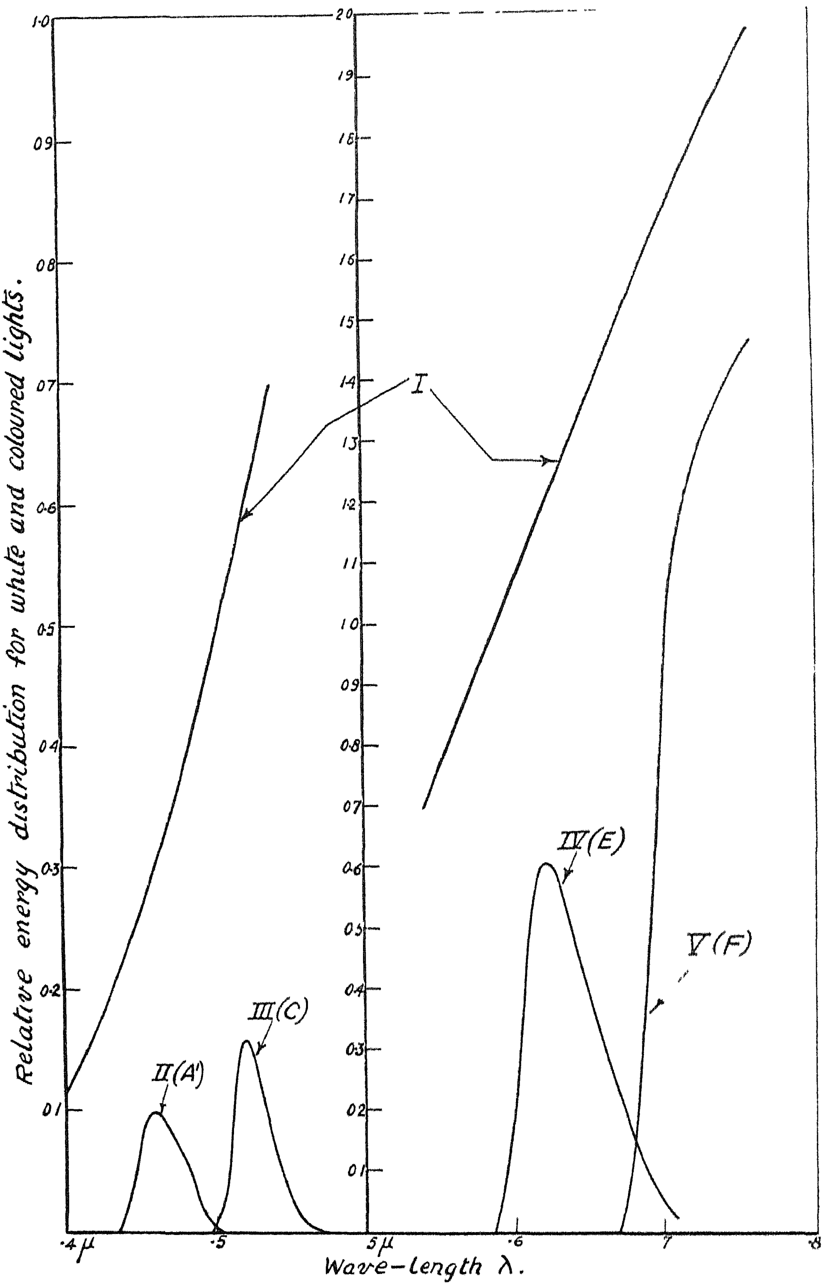

FIG. 4—Relative energy distributions. Curve I, white light from 2800 ° K source. Curves II to V coloured light obtained from 2800° K source in conjunction with filters A′, C, E, and F respectively.

known transmission in the appropriate light beams. The values given are simply the directly measured high brightness values multiplied by the factor corresponding to the transmission of the filters or diaphragms or to the increase of source distance evaluated by the inverse square law. For high white light brightnesses, the direct measurements of different observers are practically the same and our specification of both high and low white light brightnesses is therefore unique. For the coloured lights, however, the direct measurements of different observers made at high brightnesses may differ considerably (see Paper I, p. 504).

In specifying coloured light stimulations, the brightness values here used are based on the high brightness measurements made by the subject whose l.b.i. values are in question. Thus one and the same coloured conditioning stimulation is represented by a different brightness when applied to subject W. S. S. than when applied to subject B. H. C. The difference is only of note for filters A′ and F for which it amounts to 30 or 40%.

By "high brightness" must be understood a brightness sufficiently high for the photometric measurement to be unaffected by the Purkinje effect, it being assumed that the photometer used has not too large a field size (not exceeding 2° in diameter).

In all the measurements given in this paper, the pupil of the subject's eye was left free, *i.e.*, no mydriatic was employed, and an artificial pupil was never used. Also, with the exception of two sets of runs in Section 7, no lens was inserted between the subject's eye and the test spot so that if the latter was to be seen in sharp focus, accommodation by the subject was necessary. In apparatus I the test spot was formed at a distance of about 60 in. from the subject and in apparatus II at a distance of about 24 in. For the latter apparatus there is some doubt as to whether the subject actually does accommodate correctly when observing foveally in certain conditions of stimulation (low brightness level or in the presence of glare). In foveal observation it is hardly possible to use an ordinary fixation mark because this would interfere with the test spot. Orientation marks consisting of weak point sources of light, placed 2 or 3 degrees on either side of the test spot were sometimes used and seemed helpful. It would probably be advantageous in future work to place a lens in front of the eye to remove the apparent position of all objects in the field of view to infinity. This would enable the subject to see these objects sharply in focus when the eye was relaxed for distant vision. For parafoveal or peripheral observation, a fixation point must certainly be used to define the direction of vision, and at the same time it aids accommodation.

Bearing in mind Allen's (1923) work on the coupling between the retinae of the two eyes, it was desirable to define carefully the condition of the non-observing eye when using monocular vision of the test spot. Three methods were tried: (*a*) the idle eye was kept in total darkness throughout any series of measurements; (*b*) a small opaque disc was placed so as to block out the test spot from the view of the idle eye but leaving the rest of the field visible; and (*c*) a piece of very lightly ground glass was inserted in front of the idle eye so that the general brightness of the field was little altered, but objects became fuzzy, and in particular the test spot could not be seen until its intensity greatly exceeded the threshold value for the observing eye. It is clear that with methods (*b*) and (*c*) the idle eye is adapted to very much the same brightness distribution as the observing eye, whereas with method (*a*) the idle eye is always dark adapted. Trouble was experienced with methods (*b*) and (*c*) owing to inter-ocular rivalry, sometimes one eye taking charge and sometimes the other. There seemed to be no very great difference between the results by the various methods (an extensive study of this point was not made, however) and it was decided to use method (*a*) exclusively.

3—*Study of Special Points bearing on l.b.i. Measurements*

(*a*) *Effect of Speed of Appearance or Disappearance on the l.b.i. for a Continuously exposed Test Spot*—With the aid of the motor-driven lamp carriage of apparatus I it was possible to study the effect of speed of appearance, or disappearance, on the l.b.i. for a continuously exposed test spot.

The subject used binocular and foveal vision and viewed the appearance or disappearance of a circular test spot of $1 \cdot 6°$ angular diameter, projected on to a continuous background of uniform brightness. Two background brightness levels were worked with, a high brightness level of $0 \cdot 5$ candles/sq. ft. and a low brightness level of $0 \cdot 0035$ candles/sq. ft. Observations were made at each of the four principal carriage speeds corresponding to the values of $\frac{1}{u}\frac{du}{dt}$ given above. The carriage was started at a point on the bench at which the subject could easily see the test spot. When, in the subject's judgment, the test spot had disappeared, he pressed the tapping key in the cabin and a mark was recorded on the paper tape placed along the bench. The carriage was then run in the reverse direction from a point at which the test spot brightness was considerably lower than the l.b.i. and again the subject pressed the key when he judged the spot to be just visible. The electric recorder was

arranged to show a red mark on the tape for observations made with the carriage approaching and a black mark for observations made with the carriage receding from the plane F. A set of five disappearance and five appearance readings was obtained for each carriage speed and from these the mean disappearance l.b.i. T_d, and the mean appearance l.b.i. T_a, were computed.

In Table I the results are summarized. The values of T_a and T_d are in arbitrary units and the results are therefore relative only. Each row of figures represents the mean values of T_a and T_d for a determination at each of the four speeds.

Table I

Background brightness = 0·5 candles/sq. ft. Rate of change of test spot brightness $= \frac{1}{u}\frac{du}{dt}$

$\frac{1}{u}\frac{du}{dt} =$	$2{\cdot}42 \times 10^{-2}$		$6{\cdot}10 \times 10^{-2}$		$13{\cdot}8 \times 10^{-2}$		$19{\cdot}1 \times 10^{-2}$	
Subject	T_a	T_d	T_a	T_d	T_a	T_d	T_a	T_d
W. S. S. .	1·64	2·32	2·10	1·68	1·81	1·40	2·15	1·04
B. H. C. .	1·70	1·86	1·33	1·52	1·95	1·27	2·22	1·77
W. S. S. .	1·55	1·51	1·55	1·34	1·82	1·06	2·60	0·86
B. H. C. .	1·61	1·28	2·28	1·50	2·52	1·22	1·97	0·90
C. D. .	1·44	1·73	2·08	1·86	2·06	1·57	2·13	1·49
Means .	1·59	1·74	1·87	1·58	2·03	1·30	2·22	1·21
$T = \frac{T_a + T_d}{2}$	1·66		1·72		1·66		1·72	
$\frac{T_a - T_d}{T_a + T_d}$	−0·045		+0·084		+0·220		+0·294	

Background brightness = 0·0035 candles/sq. ft. Rate of change of test spot brightness $= \frac{1}{u}\frac{du}{dt}$

$\frac{1}{u}\frac{du}{dt} =$	$2{\cdot}42 \times 10^{-2}$		$6{\cdot}10 \times 10^{-2}$		$13{\cdot}8 \times 10^{-2}$		$19{\cdot}1 \times 10^{-2}$	
Subject	T_a	T_d	T_a	T_d	T_a	T_d	T_a	T_d
W. S. S. . . .	1·25	1·60	1·43	1·22	1·79	1·02	2·05	1·01
B. H. C. . . .	1·64	1·55	1·66	1·39	1·66	1·07	2·10	0·94
C. D.	1·75	1·34	1·66	1·70	1·94	1·19	2·27	1·04
B. H. C.	1·56	1·44	1·76	1·23	1·12	0·87	2·44	1·11
C. D.	1·57	1·53	1·86	1·42	1·76	1·16	1·63	1·29
Means	1·54	1·49	1·67	1·39	1·85	1·06	2·10	1·08
$T = \frac{T_a + T_d}{2}$	1·52		1·53		1·46		1·59	
$\frac{T_a - T_d}{T_a + T_d}$	+0·016		+0·092		+0·270		+0·320	

It is clear that at the lowest speed $\left(\frac{1}{u}\frac{du}{dt} = 2{\cdot}42 \times 10^{-2}\ \text{sec}^{-1}\right)$ the appearance and disappearance l.b.i.'s are not appreciably different, but as the speed is increased the appearance l.b.i. increases, the disappearance l.b.i. decreases. At both brightness levels, however, the mean l.b.i. $\left(T = \frac{T_a + T_d}{2}\right)$ is approximately independent of the rate of change of the test spot brightness. This is a

fortunate circumstance because for many purposes it is the mean l.b.i. in which we are interested and in such cases, provided the rate of change of the test spot brightness is the same for appearance and disappearance readings, the absolute value is immaterial.

The cause of the variation of T_a and T_d with rate of change of the test spot brightness is of interest. The explanation is possibly to be obtained on lines such as the following. Suppose the test spot appears or disappears at a given brightness u_T and let there be a lag of τ seconds before the subject operates the key. During the period τ the test spot brightness will have changed and what we record as T_a and T_d will be given by

$$T_a = u_T e^{+r\tau}$$

$$T_d = u_T e^{-r\tau},$$

where

$$r = \frac{1}{u}\frac{du}{dt}.$$

Thus

$$\frac{T_a - T_d}{T_a + T_d} = \frac{e^{+r\tau} - e^{-r\tau}}{e^{r\tau} + e^{-r\tau}} = \tanh r\tau$$

$$= \text{approximately } r\tau,$$

provided $r\tau$ is not too large (error of 8% when $r\tau = 0 \cdot 5$).

Thus $\frac{T_a - T_d}{T_a + T_d}$ plotted against r should give a straight line through the origin, of gradient τ. In fig. 5 the values of $\frac{T_a - T_d}{T_a + T_d}$ derived from the data given in Table I are plotted against r, together with the straight line through the origin which best fits the points. This line corresponds to $\tau = 1 \cdot 6$ sec and if our explanation is valid we may say that the subject's "period of indecision" is of that order. Our explanation also requires that $T = \frac{T_a + T_d}{2}$ should be independent of r for not too large values of $r\tau$, a requirement with which our experimental results comply.

(*b*) *Use of a Flashing Test Spot. Single Decision and Multiple Decision Methods for Determining the l.b.i.*—When a continuously exposed test spot is observed by extrafoveal vision, the determination of the l.b.i. is complicated by the well-known effect that on prolonged fixation objects viewed extrafoveally tend to disappear. Fig. 6 shows a set of observations of the l.b.i. made with apparatus I, in which the subject fixated on a small point of light separated from the $1 \cdot 6°$ diameter circular test spot by an angle varying from 2° to

25°. Binocular vision was used with a uniform background of about 0·1 candles/sq. ft. The figure shows both the appearance and disappearance thresholds. In passing out from the fovea centralis the l.b.i. steadily increases to about five times its initial value, but owing to the effect just noted the appearance

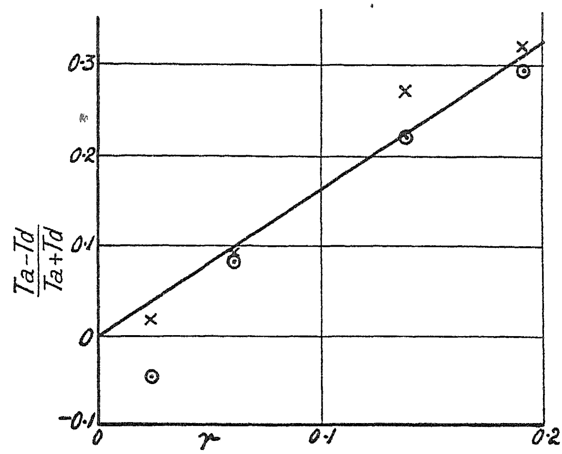

FIG. 5—× Background brightness = 0·0035 c/sq. ft.; ⊙ background brightness = 0·5 c/sq. ft.

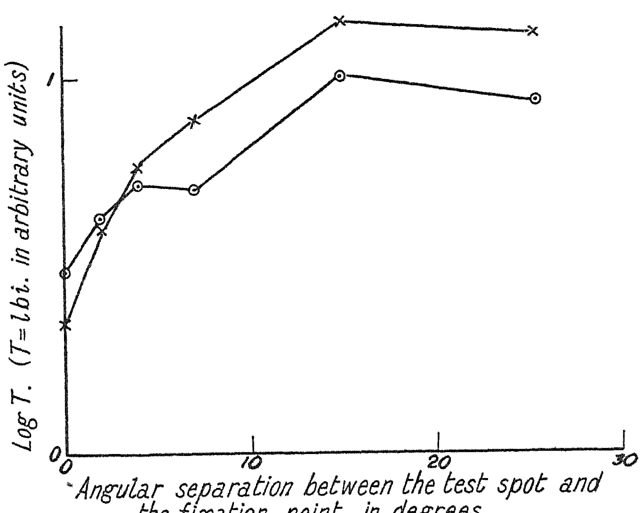

FIG. 6—⊙ T_a appearance l.b.i.; × T_d disappearance l.b.i.

and disappearance l.b.i.'s cross over at about 3°. To avoid this complication, when making extrafoveal readings the test spot instead of being exposed continuously was exposed periodically for a definite flash period by means of the shutter at K. Experiment shows that with foveal observation the l.b.i.

for a flash period of about 1 sec or more is practically the same as for a continuously exposed test spot. Extrafoveal observations with such a flash period are therefore comparable with foveal observations obtained for a continuously variable test spot.

The use of a flashing test spot led to a modification in the method of taking readings. Instead of requiring the subject to signal when he judges that the test spot is visible or invisible, he is asked to signal whether he has or has not seen each individual exposure of the test spot. He is supplied with two keys: one marking red on the bench tape, the other black, and he depresses the red key for "seen," the black key for "not seen." The record obtained on the tape then consists, for a disappearance reading, of a section of red dots followed by a region in which both black and red dots occur, and finally of a section of black dots. The value of the l.b.i. is taken to correspond to the point on the record such that there are as many red dots on the main black dot series side as there are black dots on the red dot series side. This corresponds crudely to the following definition of the l.b.i.:

T is such that on the average for values of $\log u$ less than $\log$ T the test spot is as likely to be seen as it is likely to be missed for values of $\log u$ greater than $\log$ T.

Using this method, appearance and disappearance l.b.i.'s show no significant difference, at any rate for the fairly slow rates of variation of the test spot brightness which we have used. The subject is relieved from the strain of making a single decision on the correctness of which the value of a determination will depend. He has, instead, to make a number of minor decisions, and an error in one of these will not seriously affect the result. This method, which may be termed the "multiple decision method," has proved very satisfactory in use. It will be convenient to refer to the earlier method as the "single decision method."

(c) *Time for the Eye to reach a Steady State and Variability of l.b.i. Readings*

In a previous investigation (Stiles, 1929) it was shown that measurements of the l.b.i. are subject to variations of two kinds: (i) chance error variations representing deviations from the "instantaneous" l.b.i. value, and (ii) variations due to changes of the instantaneous l.b.i. value from time to time. Variations of the latter type may be large compared with the chance errors, and their origin is obscure. It was believed that the previous light history of the eye might be the cause. If so, it might be possible to eliminate variations of this type by standardizing the previous light history of the eye. An extended series of measurements was made to test this possibility. The

method tried was to expose the eyes for some time to a very high brightness which, it was hoped, would swamp any carry-over of effects from previous stimulations.

Using apparatus II, the subject, employing monocular vision, was required to look steadily at a white background of brightness 180 candles/sq. ft. for a period of 10 minutes. At the end of this time the brightness of the background was rapidly changed to a new value B_F (always lower than 180 candles/sq. ft.) and readings were taken of the foveal and 5°-parafoveal l.b.i., using the single decision method, at intervals for a period of about 70 minutes. The values chosen for the background brightness B_F were approximately the following: 16, 4·2, 0·063, 0·00046, and 0 candles/sq. ft. For each value two or three runs were obtained for two subjects. For each subject only one run was made in one day.

The values of the l.b.i. for the subject B. H. C. at various times after the initial few minutes, obtained as means from the several runs under identical conditions, are shown in figs. 7 and 8.

For both foveal and parafoveal readings there is no very clearly defined systematic change in the mean values of the l.b.i. in the period within which readings were taken, except for $B_F = 0$ (foveal readings) and for $B_F = 0 \cdot 0005$ or 0 (parafoveal readings). Let us omit from consideration for the moment the last-mentioned conditions. Take now the values of log T obtained at different times in any single run. Each such value is the mean of one appearance and one disappearance observation by the single decision method, and we may regard it in the statistical sense as a single observation. The scatter of these values will certainly not be less than the scatter corresponding to chance error deviations from an instantaneous l.b.i. value, because, besides the latter deviations, the observed scatter will include deviations due to possible fluctuations in the instantaneous l.b.i. value during the course of a run. The probable error of a single observation of log T derived from all the observations in a single run has been calculated, by an approximate method, for each run, and we shall take this value as an upper limit to the probable error of a single observation due to random deviations from an instantaneous l.b.i. value. The probable error of the arithmetic mean of the observations of log T in a single run is obtained from the probable error of a single observation by dividing by the square root of the number of single observations included in the arithmetic mean.

In Table II the first column gives the measured value of log B_F for each run, and the second column gives the arithmetic mean of all observations of $\log_{10}$ T

in each run. The probable errors of a single observation and of the arithmetic mean of $\log_{10} T$ are shown in columns 3 and 4. The values of $\log B_F$ in the two or three similar runs are not quite the same and as we wish to obtain the changes in $\log_{10} T$ in different runs under identical conditions it is necessary

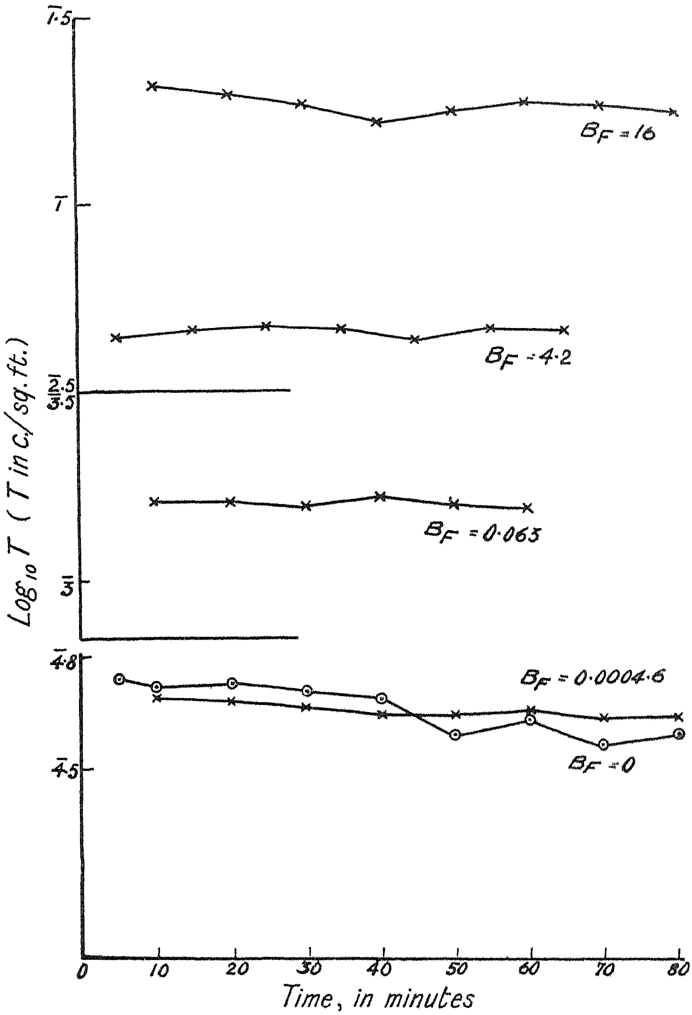

FIG. 7—Foveal readings, subject B. H. C.

to reduce the observed values of $\log_{10} T$ to values appropriate to a common B_F. This was done by plotting $\log T$ against $\log B_F$ over the whole range of $\log B_F$ (from $1\cdot2$ to $\bar{4}\cdot6$) and determining from the curve the rate of change of $\log T$ with respect to $\log B_F$ at the values of $\log B_F$ at which the different groups of runs were made. The arithmetic means of $\log T$ reduced to a common

value of log B_F are shown in column 5, and column 6 gives the differences between the reduced arithmetic means of log T in successive runs.

Examining this table we see that the average values of the probable error of a single observation of $\log_{10}$ T (column 3) are as follows: 0·027 (fovea), 0·032 (parafovea). For another subject (F. W. C.) the values were 0·033 and

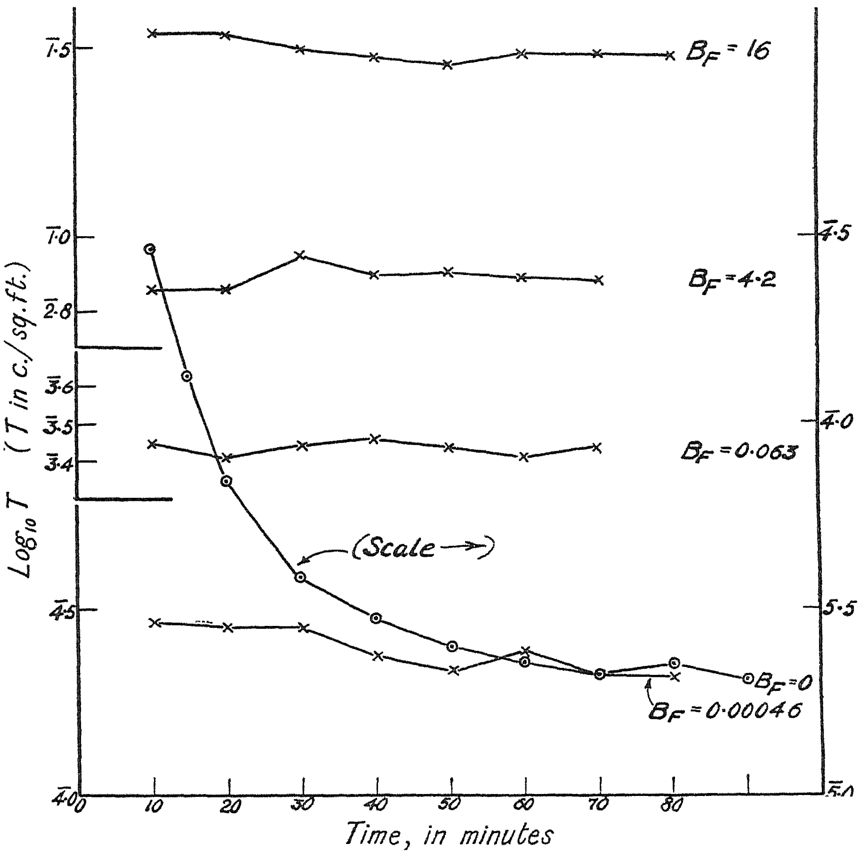

Fig. 8—5°-parafoveal readings, subject B. H. C.

0·040 (parafovea). Errors of 0·027, 0·032, 0·033, and 0·040 in log T correspond respectively to errors of 6·4, 7·6, 7·9, and 11·0% in T. In the previous investigation (Stiles, 1929) it was estimated that the probable error at a single observation of T by the single decision method equalled 7%. Thus the present results are in substantial agreement with this estimate.

Turning now to the differences between the reduced arithmetic mean values of log T obtained in successive runs (column 6) we see that for B. H. C. the average value of these differences equals 0·074 (fovea), 0·094 (5°-parafovea).

Table II—Subject, B. H. C.

Log B_F	Arithmetic mean of log T	Probable error of single observation of log T	Probable error of A.M. of log T		A.M. of log T reduced to common value of log B_F	Deviations between log B_F reduced A.M. of log T in successive runs
			Foveal Readings.			
1·255	$\bar{1}\cdot 34$	0·041	0·014		$\bar{1}\cdot 28$	0·02
1·212	$\bar{1}\cdot 27$	0·030	0·009	(log B_F = 1·200)	$\bar{1}\cdot 26$	0·07
1·167	$\bar{1}\cdot 15$	0·030	0·010		1·19	
0·627	$\bar{2}\cdot 69$	0·014	0·005		$\bar{2}\cdot 68$	0·06
0·627	$\bar{2}\cdot 63$	0·026	0·009	(log B_F = 0·620)	$\bar{2}\cdot 62$	0·07
0·600	$\bar{2}\cdot 67$	0·034	0·012		$\bar{2}\cdot 69$	
$\bar{2}\cdot 875$	$\bar{3}\cdot 12$	0·019	0·007		$\bar{3}\cdot 08$	0·12
$\bar{2}\cdot 799$	$\bar{3}\cdot 20$	0·023	0·008	(log B_F = $\bar{2}\cdot 800$)	$\bar{3}\cdot 20$	0·13
$\bar{2}\cdot 716$	$\bar{3}\cdot 28$	0·021	0·007		$\bar{3}\cdot 33$	
$\bar{4}\cdot 690$	$\bar{4}\cdot 68$	0·032	0·011	(log B_F = $\bar{4}\cdot 66$)	$\bar{4}\cdot 68$	0·05
$\bar{4}\cdot 643$	$\bar{4}\cdot 63$	0·030	0·011		$\bar{4}\cdot 63$	
	Mean	0·027	0·0094		Mean	0·074
			5°-parafoveal Readings.			
1·255	$\bar{1}\cdot 62$	0·058	0·019		$\bar{1}\cdot 56$	0·10
1·212	$\bar{1}\cdot 47$	0·030	0·009	($\log_{10} B_F$ = 1·200)	$\bar{1}\cdot 46$	0·03
1·167	$\bar{1}\cdot 34$	0·029	0·010		$\bar{1}\cdot 43$	
0·627	$\bar{2}\cdot 85$	0·033	0·011	($\log_{10} B_F$ = 0·620)	$\bar{2}\cdot 84$	0·11
0·600	$\bar{2}\cdot 93$	0·044	0·006		$\bar{2}\cdot 95$	
$\bar{2}\cdot 875$	$\bar{3}\cdot 37$	0·020	0·008		$\bar{3}\cdot 33$	0·09
$\bar{2}\cdot 799$	$\bar{3}\cdot 42$	0·020	0·007	($\log_{10} B_F$ = $\bar{2}\cdot 800$)	$\bar{3}\cdot 42$	0·14
$\bar{2}\cdot 716$	$\bar{3}\cdot 51$	0·020	0·007		$\bar{3}\cdot 56$	
	Mean	0·032	0·0096		Mean	0·094

For F. W. C. the values are 0·078 and 0·070 respectively. Thus a redetermination under identical conditions gave a mean value of log T which on the average differed from the original mean value by 0·079, *i.e.*, the value of T differed by 20%. Now, owing to the chance errors to which the determination of the instantaneous l.b.i. is subject, we should expect the mean values obtained in different runs to vary, and it is easy to show that if the instantaneous l.b.i. remains unchanged, then the average difference between the mean results of two runs must equal 0·944 times the probable error of the mean value of a single run. In Table III the ratio of the average difference between the arithmetic means of successive runs to the average probable error of the mean value of a single run is shown for the foveal and parafoveal data of the two subjects. The value of this ratio ranges from 5·0 to 9·8 and in every case it far exceeds 0·944. We may conclude therefore that practically the whole of the variation

between the successive determinations of the l.b.i. is due to change in the instantaneous l.b.i. value (what has been termed previously " drift variation ").

Thus standardizing the previous light history of the eye by an initial 10 minutes exposure to a high brightness has failed to eliminate the large variations of the instantaneous l.b.i. value which have constantly been observed in this work. It is possible that these variations are unconnected with the light history of the eye. They may arise by physiological changes in the subject or psychologically by a change in the subject's criterion of visible and invisible.

Table III

		B. H. C.		F. W. C.	
		Fovea	Parafoveal	Fovea	Parafoveal
Average difference between—A.M.s' of successive runs / probable error of A.M.	=	7·9	9·8	6·6	5·0
		Mean value = 7·3			

There is one further piece of evidence which can be extracted from this set of data. At each sitting (with one or two exceptions) both a foveal and a 5°-parafoveal run was obtained. Thus we can determine for successive sessions under identical conditions the direction and amount of the change in

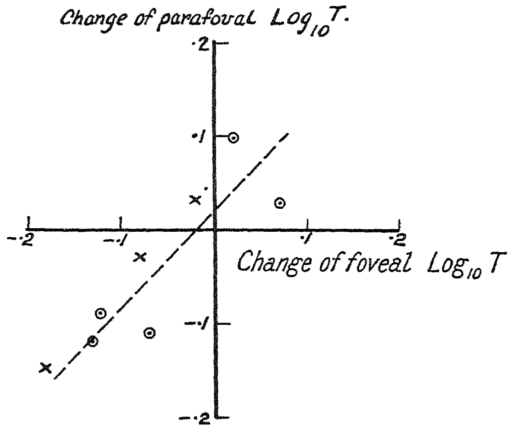

FIG. 9—⊙ B. H. C.; × F. W. C.

the mean foveal and mean parafoveal readings. These data for B. H. C. are, in fact, given in the sixth column of Table II, account being taken of the sign placed after the numeric. In fig. 9 the difference between the parafoveal values of log T in successive runs under identical conditions is plotted against the difference between the corresponding foveal values. It is clear that to a

rough approximation a change in the foveal l.b.i. is accompanied by a change in the parafoveal l.b.i. in the same sense and of about the same amount. Thus the cause of the variations, whatever it may be, must operate similarly in the fovea and parafovea.

The experiments not considered in detail, $B_F = 0$ (foveal readings) and $B_F = 0 \cdot 0005$ or 0 (parafoveal readings), in which a systematic change of l.b.i. with time takes place after switching off the initial high brightness field, bear out the conclusions already obtained.

(*d*) *Conditions in the Dark Centre Field*— When using a dark circular centre field surrounded by a bright outer field, within which the test spot appears, the circular form of test spot is unsuitable. This is because, on prolonged fixation of the circular dark area in a bright surround, a haze of light concentric with the centre field tends to form, which is easy to confuse with the test spot. Test spots of various shapes were tried, the most generally satisfactory form seemed to be a rectangle $0 \cdot 70° \times 0 \cdot 16°$. We also tried making the test spot rotate slowly while fading in or out. Various sets of data obtained with different test spots are shown in fig. 15.

As will be seen from the data to be given later, the l.b.i. for a test spot seen in the centre of the dark centre field, surrounded by a brightness B_S, is in general much smaller than the l.b.i. for the same test spot seen against a uniform background of brightness B equal to B_S. The question then arises: is the difference due to the fact that the patch of retina corresponding to the dark centre field is in a different state of adaptation than the surrounding area (alternative (*a*)), or is it due to the fact that the test spot is seen against zero brightness with the surround, and against a brightness $B = B_S$ with the background (alternative (*b*))? If the latter alternative is true, then on changing the conditioning stimulation abruptly from the surround to the background case, or *vice versa*, the l.b.i. should also change abruptly. If, however, a change in the state of adaptation of the central area is involved, then this presumably must take time to occur, and the l.b.i. should pass more or less gradually from the surround to the background value or *vice versa*.

The following experiment was made to test this point. Using apparatus II the subject viewed by monocular foveal vision a uniform background of brightness B = approx. $0 \cdot 5$ candles/sq. ft. Every 4 seconds a trap door in the background screen was made to open sharply, disclosing a $1 \cdot 28°$ diameter centre field of zero brightness. The trap door remained open for 1 second and was then closed, thereby restoring the uniform background. At a given

instant in this 4 seconds cycle the test spot (rectangular, $0{\cdot}7^\circ \times 0{\cdot}16^\circ$) was flashed for 1/10th of a second on to the centre of the test field. If the instant chosen occurred in the 1 second interval during which the trap door was open, the test spot was superposed on zero brightness, in other cases it was superposed on the brightness B. The l.b.i. measurement was made by the single decision method, the intensity of the test spot flash being increased by a fixed proportion at each exposure until the subject signalled "visible," and then decreased in the same way until the subject signalled "not visible."

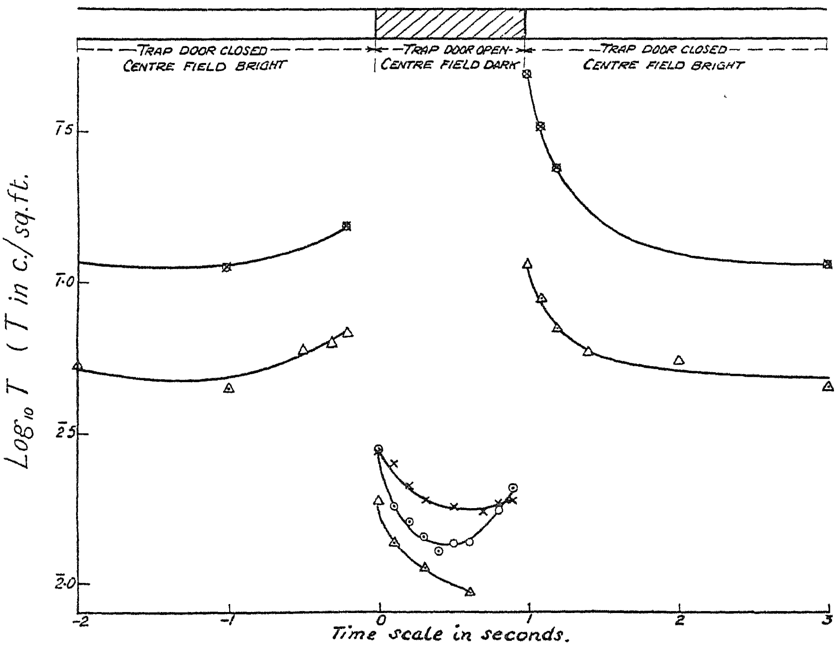

FIG. 10—△ W. S. S.; ⊙ W. S. S.; × B. H. C.

Fig. 10 shows the results obtained. The data fit in with neither of the simple hypotheses indicated above (see fig. 11). It appears that just before the door is closed or opened the l.b.i. begins to rise. This may be connected with the use of a cyclic method in which the subject knows exactly what is going to happen, or it may be due to the fact that the powerful sensation corresponding to the opening or closing of the trap-door "overtakes" and partially nullifies the weak test spot sensation. The l.b.i., immediately after the trap-door is closed, attains a value much higher than that corresponding to the steady background. This again points to a kind of interference between the sensation corresponding to the abrupt change of conditioning stimulation, and the test

spot sensation. Further results which have been obtained on effects of this kind are reserved for a later paper.

Another attempt to throw light on the question of conditions in the dark centre field is represented by fig. 12 which shows the mean values of log T for foveal vision obtained with a small 0·1° diameter test spot placed at different points on a diameter of a 1° circular dark field with a surround brightness $B_S = 16$ candles/sq. ft. The l.b.i. is practically constant within an inner

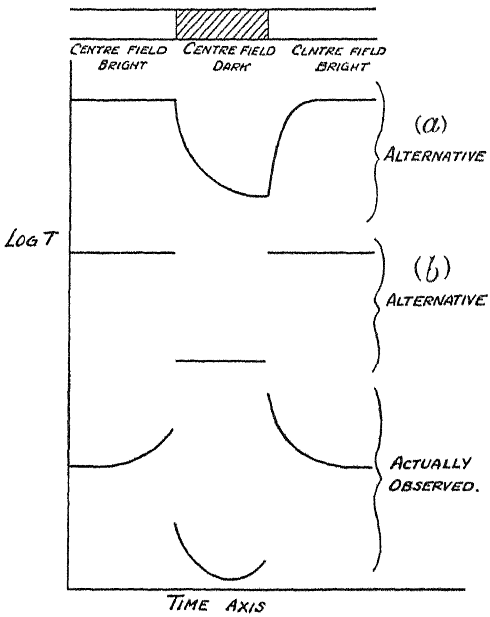

FIG. 11

circle of 0·5° diameter, beyond which it rises steadily as the edge of the dark field is approached.

(*e*) *Relative Positions of Test Spot, Fixation Point, and Glare Source in Parafoveal Measurements*—When the subject views the test spot parafoveally and the conditioning stimulation is a glare source, the question arises whether, for a fixed angular separation between glare source and test spot, the value of the l.b.i. is dependent on the angular separation between the glare source and the fixation point (direction of vision). Using apparatus I the test spot and fixation point were fixed at 5° apart and the glare source was moved round on the circumference of a semi-circle of 5° radius (*a*) with the test spot as centre, and (*b*) with the fixation point as centre (see fig. 13). In (*a*) the separation between glare source and test spot remained constant, and in (*b*) the separation

between glare source and fixation point was unchanged. The results are summarized in Table IV.

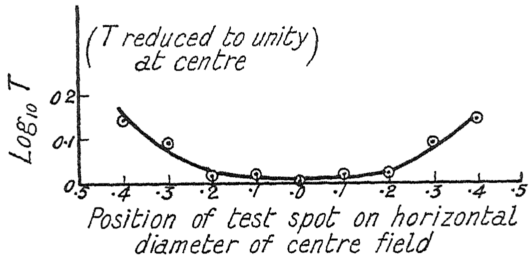

FIG. 12—Mean curve.

Table IV—Background brightness = 0. Subjects B. H. C. and F. W. C. Monocular vision. Test spot at fixed angular separation of 5° from fixation point. Illumination at subject's eye due to glare source = 4·2 f.c.

Arrangement (*a*)

Angle between glare source and fixation point	Log_{10} T B. H. C.	Log_{10} T F. W. C.
°		
0·5	$\bar{2}\cdot81$	$\bar{2}\cdot95$
1·5	$\bar{2}\cdot83$	$\bar{2}\cdot92$
3	$\bar{2}\cdot87$	$\bar{2}\cdot96$
5	$\bar{2}\cdot77$	$\bar{2}\cdot91$
8·7	$\bar{2}\cdot86$	$\bar{2}\cdot93$
10	$\bar{2}\cdot84$	$\bar{1}\cdot00$

Arrangement (*b*)

Angle between glare source and test spot	Log_{10} T B. H. C.
°	
1·5	$\bar{1}\cdot77$
3	$\bar{1}\cdot23$
5	$\bar{2}\cdot78$
7	$\bar{2}\cdot60$
10	$\bar{2}\cdot35$

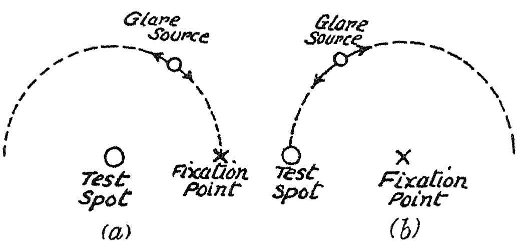

FIG. 13

It is clear from the results with arrangement (*a*), that for a constant separation between glare source and test spot, the l.b.i. is independent of the position

of the glare source with respect to the fixation point. We may perhaps interpret this as meaning that the whole effect of the glare is transmitted through the retina directly from the position of the glare source to that of the test spot, and that the glare does not operate indirectly via the fovea centralis. The measurements with arrangement (*b*) merely illustrate the very large effect on the l.b.i. when the separation between glare source and fixation point is fixed but the angle between glare source and test spot is varied.

4—*Conditioning Stimulation: White Background or White Surround. Foveal Vision of Continuously Exposed Test Spot*

Measurements of the l.b.i. for a 1° circular test spot exposed continuously and viewed against a white background of brightness ranging from 0·001 to 500 candles/sq. ft. are given in fig. 14. Normal, direct monocular vision was employed, and no special care was exercised to ensure accurate foveal fixation at low brightness levels. As, in the greater part of the brightness range the fovea is certainly more sensitive than the parafovea, the results may safely be assumed to refer to foveal vision, except possibly for the points at log B = $\bar{3}$ (B = 0·001).

It will be noted that these points are somewhat lower than would be expected if the curve proceeds in continuous fashion without change of law. As shown later, at brightnesses below log B = $\bar{3}$, the parafovea becomes more sensitive than the fovea. If accurate fixation is not insisted upon, the eye tends to turn so as to employ its greatest sensitivity, with the result that at brightnesses above 0·001 observation will be foveal, at brightnesses below 0·001, parafoveal.

From the highest brightnesses studied down to log B equals approximately $\bar{1}$·60, the graphs of fig. 14 are linear, which means that $T = FB^m$, where F and m are constants (F = 0·015, m = 0·94 (B. H. C.), F = 0·011, m = 1·05 (W. S. S.)). Thus in this region Weber's law T/B = constant is approximately obeyed. At brightnesses less than log B = $\bar{1}$·60, the gradient of the curves decreases steadily until at log B = $\bar{3}$ the final points indicate the intrusion of parafoveal vision as explained above.

For the measurements with a uniform surround brightness enclosing a dark centre field (diameter 1·28°), difficulty was experienced owing to the fact that, on continued fixation, a luminous haze (subjective) formed in the centre field. Test spots of different shapes were tried and the values obtained are plotted in fig. 15. The effect of slowly rotating the test spot was also

tried and the data are reproduced in the same figure. It appears that the shape of the curve relating log T to log B_S is rather different for the rotating test spot. Of the various test spots tried, the stationary rectangle proved most satisfactory, although it was always very difficult to make measurements with a continuously exposed test spot appearing in a dark centre field.

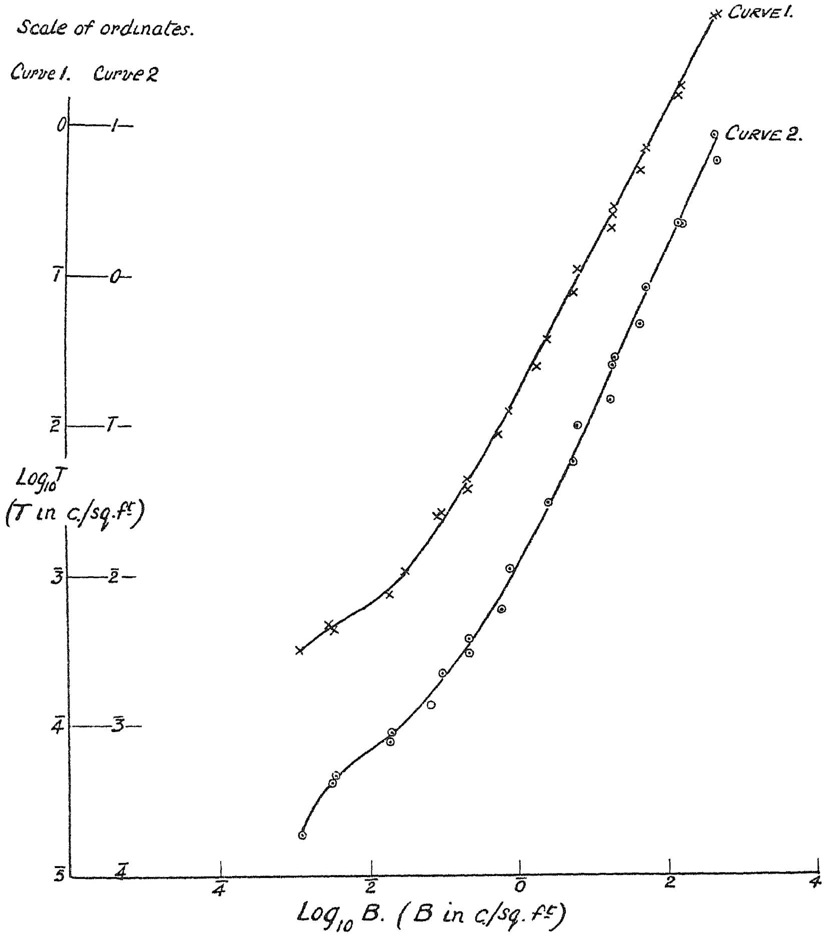

FIG. 14—Curve I, subject B. H. C.; curve 2, subject W. S. S.

The data for the surround brightnesses show much the same general characteristics as for the background brightnesses. There is an approximately linear relation between log T and log B_S at higher brightnesses and the curves then flatten out. For a given value of log T, however, the corresponding surround brightness is higher than the corresponding background brightness. For example, if log T $= \bar{1}\cdot 0$, $\log_{10} B = 0\cdot 91$ approximately (fig. 14), whereas

$\log_{10} B_S = 2 \cdot 0$ approximately (fig. 15). Thus to obtain the same value of the l.b.i. the surround brightness must in this case be of the order of 10 times the background brightness. It may be objected that the test spots used in the background and surround runs were not the same. This is, of course, true,

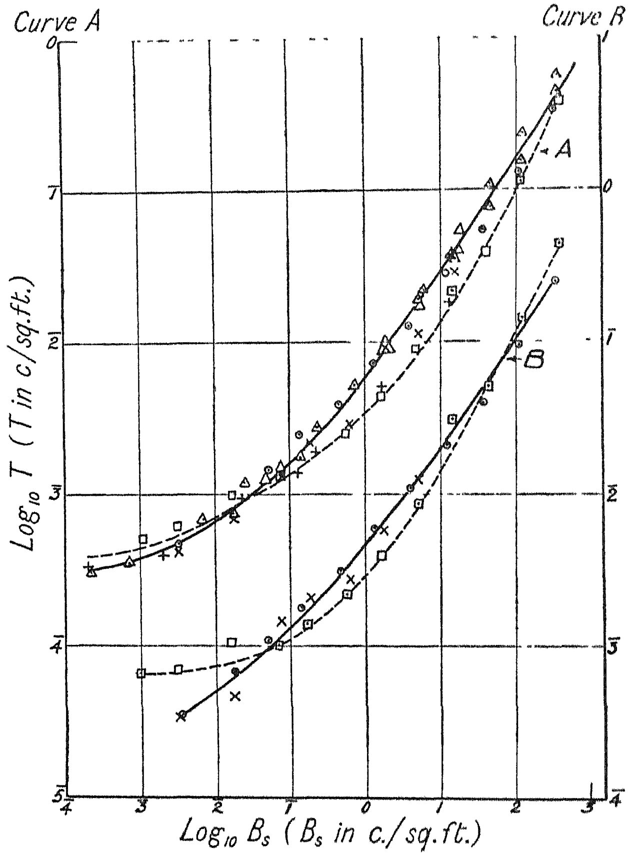

FIG. 15—Curve A, subject B. H. C.; curve B, subject W. S. S.

Test spot

Stationary, continuous curve: × Circular 0·7° diameter. ⊙ Semi-circular, 0·6° diameter. △ Horizontal rectangle, 0·7° × 0·16°.

Rotating, broken curve: ⊡ Semi-circular. + Rectangle.

but as will appear from later observations using identical flashing test spots for both background and surround brightnesses, for the fovea at high brightness levels background brightness is in fact about 10 times as effective as surround brightness in determining the value of the l.b.i.

On the other hand, the present data indicate that at lower brightnesses ($\log B$ less than $\bar{2}$) the l.b.i. values for surround and background are not very different.

5—*L.b.i. for Parafoveal Vision with White Conditioning Stimulations*

Measurements in the parafovea were made with the test spot appearing at a point situated 5° to the side of the direction of vision. As already explained, a continuously exposed test spot is unsuitable for parafoveal observations, and a flashing test spot must be employed. In figs. 16 and 17 are shown the results for two subjects of several series of observations of the 5°-parafoveal l.b.i. obtained with test spots of different sizes and flash periods viewed against white background brightnesses, covering a wide range of intensities. Before commencing a series of measurements the subject remained in total darkness for 1 hour to ensure good dark adaptation. The measurements were then taken in order of increasing background brightness so that at each brightness level the eye had not been exposed to a higher brightness for a period of at least 1 hour. The time to adapt from a lower to a higher brightness level is relatively brief, and we may assume the measurements given to apply to the eye in a steady condition of adaptation appropriate to the background brightness concerned.

Each of the curves shown in figs. 16 and 17 are the result of a single series of observations. On repeating a series of observations on another day, the results are usually found to differ to a greater or less degree. In fig. 18 are plotted the results of three runs all obtained under identical conditions and with a test spot of the same diameter and flash period. The wide variations in the l.b.i. values obtained on different days are clearly brought out in this diagram. It seems that nothing can be done to eliminate variations of this kind (*cf.* Section 3), and in any quantitative interpretation of l.b.i. measurements these day to day variations must constantly be kept in mind.

All the curves showing the variation of the 5°-parafoveal l.b.i. with background brightness have the same general shape. Starting from high brightness levels, $\log T$ decreases steadily with $\log B$ and there is a tendency for the curve to flatten out at about $\log_{10} B = \bar{3}$ or $B = 0{\cdot}001$. At lower values of $\log B$, however, the curve takes a further downward sweep and finally flattens out at about $\log_{10} B = \bar{6}$. There is apparently a change of law in the neighbourhood of $\log B = \bar{3}$ and in terms of the duplicity theory, we may associate the higher brightness range with parafoveal vision by cone mechanism and the lower

brightness range with parafoveal vision by rod mechanism. This view is confirmed by foveal measurements. For some of the series of parafoveal observations of figs. 16 and 17 concurrent foveal measurements were made,

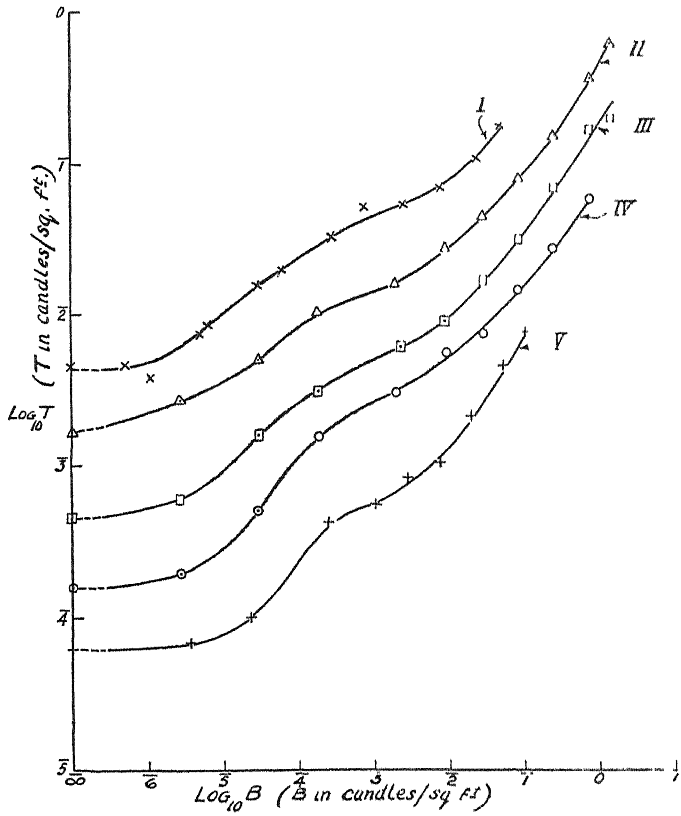

FIG. 16—Parafoveal curves obtained with different test spots:

	Angular diameter δ'	Flash period p sec
Curve I	0·07	0·05
,, II	0·11	0·05
,, III	0·11	0·75
,, IV	0·4	0·05
,, V	0·4	0·75

(Subject B. H. C.)

and these are shown for one test in fig. 19 together with the corresponding parafoveal data. It is apparent that in the higher brightness range, foveal and parafoveal curves run parallel, the parafoveal l.b.i. being greater than the foveal value. Below $\log B = \bar{3}$, however, the foveal curve has flattened out

whereas the parafoveal curve crosses the foveal curve and terminates finally at a much lower l.b.i. value than for the fovea. Similar relations hold good for the other comparisons between foveal and parafoveal curves, not shown here. Accepting that the rod mechanism is absent at the fovea, the parallelism of the foveal and parafoveal curves for B $>$ 0·001 connects the upper range of the parafoveal curve with cone mechanism, and the constancy of the foveal

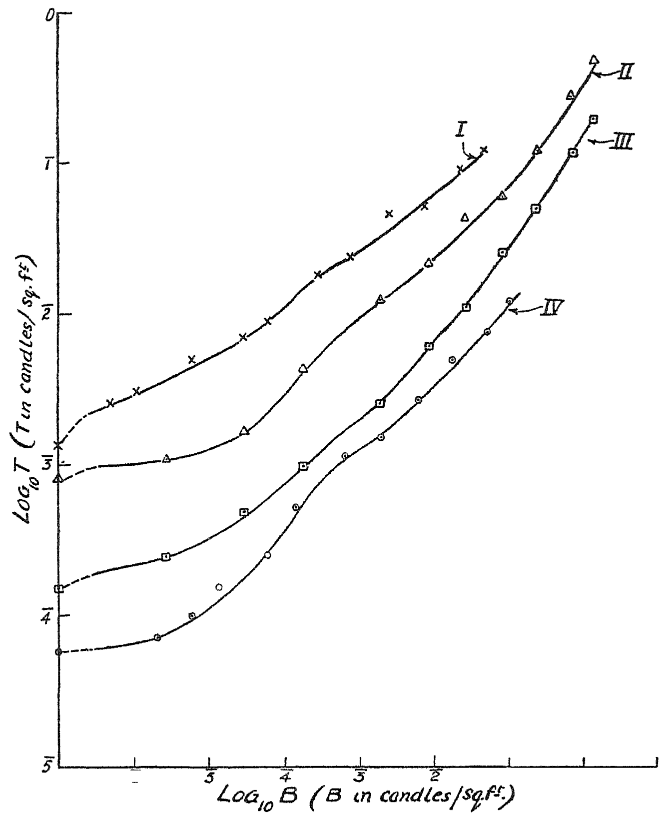

FIG. 17—Subject W. S. S. Key as in Fig. 16.

l.b.i. for B $<$ 0·001 connects the lower part of the parafoveal curve with rod mechanism.

The following differences between the data for the two subjects should be noted. The change over from the high brightness (B $>$ 0·001) to the low brightness range of the parafoveal curves is, as a rule, much less marked for W. S. S. than for B. H. C. The parafoveal l.b.i. is always lower for W. S. S. than for B. H. C., the actual values for zero brightness being in a ratio of the order 1 : 3.

For a conditioning stimulation consisting of a bright patch of light (white) in an otherwise dark field, fig. 20 shows two sets of observations obtained for 5°-parafoveal vision with the glare source placed 6° above the test spot. Log T is plotted against $\log_{10}$ E where E is the illumination of the subject's eye due to the glare source. The curves resemble those showing the variation of log T with log B and exhibit the same characteristic change of law. In fact,

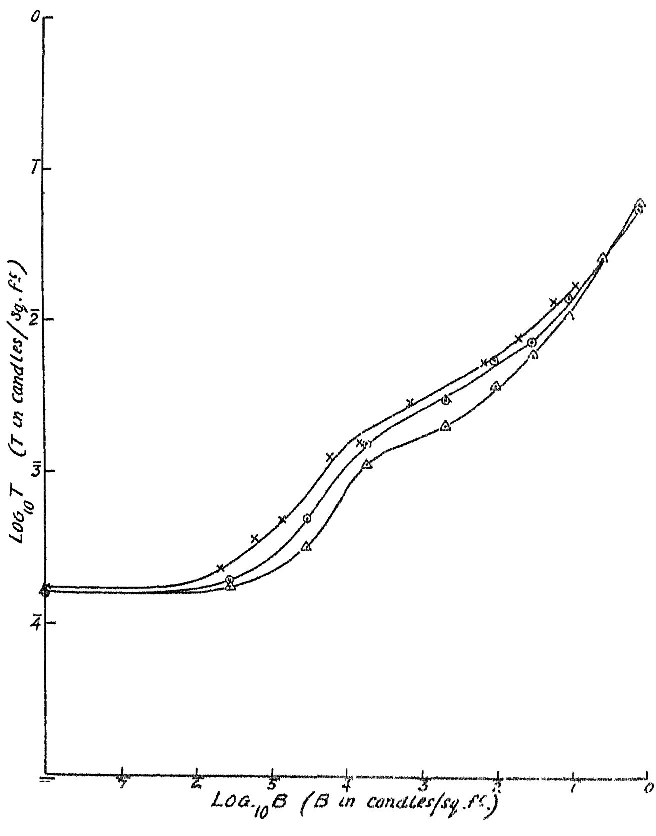

FIG. 18—Parafoveal curves for the same test spot (δ = 0·4°, p = 0·05 sec) obtained on different occasions.

curve I of fig. 20 can be brought into approximate coincidence with curve V of fig. 17 obtained with the same test spot, by a displacement parallel to the axis of abscissæ, the agreement being about as good as that obtainable between redeterminations of the individual curves. By noting the amount of displacement required, we obtain the following result: a glare source at 6° above the test spot and producing an eye illumination of E ft. c. gives the same l.b.i. for 5°-parafoveal vision as a uniform background of brightness β equal to

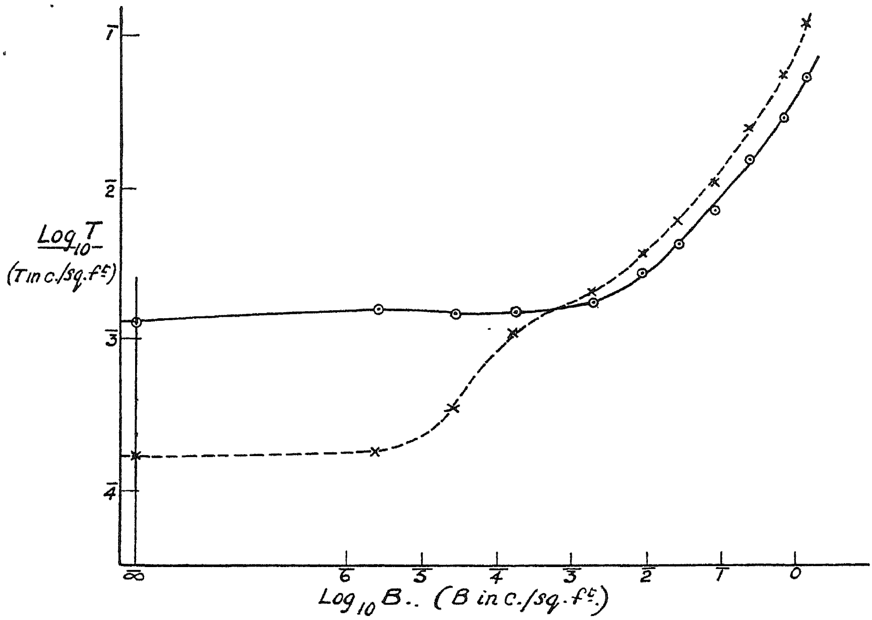

FIG. 19—Foveal and parafoveal curves for the same test spot ($\delta = 0{\cdot}4°$, $p = 0{\cdot}05$ sec).

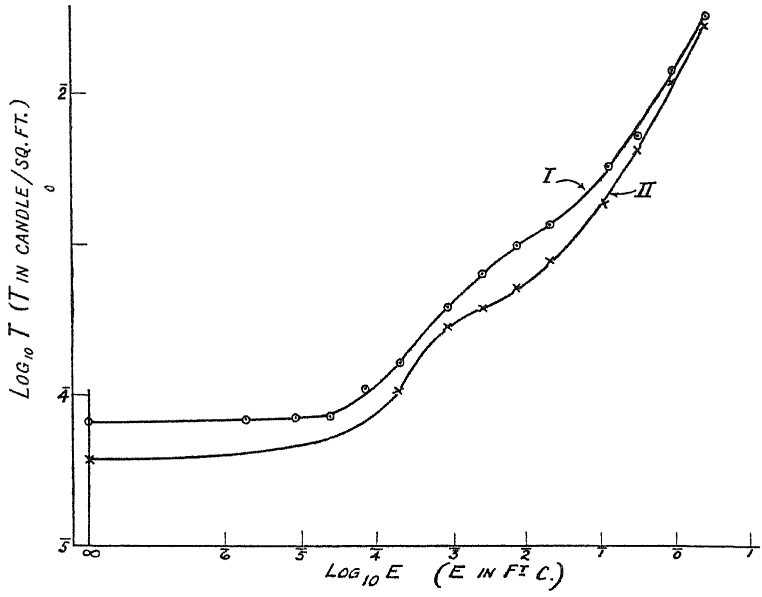

FIG. 20—Curve I, B. H. C.; curve II, W. S. S. Test spot, $\delta = 0{\cdot}4°$, $p = 0{\cdot}75$ sec.

0·3 E candles/sq. ft. β is termed the equivalent background brightness of the given glare condition.

Determinations of the l.b.i. for parafoveal vision when the conditioning stimulation was a uniform surround brightness with the test spot appearing in the centre of the dark centre field proved difficult, especially at low brightnesses and for some subjects. We shall refer to this point in the next section.

6—*Foveal and Parafoveal l.b.i. Values over Restricted Brightness Ranges*

In an investigation (Stiles and Crawford, 1932) to discover whether the equivalent background brightness of a given glare condition was dependent on the character of the test spot used in its determination, numerous measurements of the l.b.i. for different test spots over restricted ranges of background and surround brightnesses and for two or three glare source conditions were obtained. The data now to be given represent for each type of test spot (δ = angular diameter, p = flash period) and for each range of background or surround brightness and for each glare condition the mean results of three or four runs made on different days. The results for three subjects (B. H. C., W. S. S., F. W. C.) are shown in Tables V to VII. In figs. 21-23 the data for background and surround brightnesses are plotted for each of the three subjects.

Table V—Values of $\log_{10}$ T. Conditioning Stimulation: Uniform Background Brightness B

Subject B. H. C.

$\log_{10}$ B	Test spot*: $p = 0\cdot75$ $\delta = 0\cdot11$	$p = 0\cdot05$ $\delta = 0\cdot11$	$p = 0\cdot05$ $\delta = 0\cdot40$
Fovea			
0·83		0·00	$\bar{1}$·11
0·35		$\bar{1}$·73	$\bar{2}$·97
0·01	$\bar{2}$·77	$\bar{1}$·41	$\bar{2}$·61
$\bar{1}$·74	$\bar{2}$·58	$\bar{1}$·27	$\bar{2}$·46
$\bar{1}$·46	$\bar{2}$·46	$\bar{1}$·10	$\bar{2}$·32
$\bar{1}$·12	$\bar{2}$·26	$\bar{2}$·86	$\bar{2}$·06
Parafovea			
$\bar{1}$·99	$\bar{1}$·32	$\bar{1}$·74	$\bar{1}$·01
$\bar{1}$·69	$\bar{1}$·04	$\bar{1}$·48	$\bar{2}$·77
$\bar{1}$·41	$\bar{2}$·88	$\bar{1}$·30	$\bar{2}$·53
$\bar{1}$·07	$\bar{2}$·49	$\bar{1}$·07	$\bar{2}$·27
$\bar{4}$·40	$\bar{3}$·51	$\bar{2}$·23	$\bar{3}$·12
$\bar{5}$·97	$\bar{3}$·39	$\bar{2}$·00	$\bar{4}$·89
$\bar{5}$·67	$\bar{3}$·22	$\bar{3}$·86	$\bar{4}$·66
$\bar{5}$·36	$\bar{3}$·04	$\bar{3}$·70	$\bar{4}$·50
$\bar{5}$·06	$\bar{4}$·91	$\bar{3}$·51	$\bar{4}$·33

* In Tables V, VI and VII, as elsewhere, p and δ are expressed in seconds and degrees respectively.

Table V—(continued)

Subject W. S. S.

$\log_{10} B$	Test spot: $p = 0\cdot75$ $\delta = 0\cdot11$	$p = 0\cdot05$ $\delta = 0\cdot11$	$p = 0\cdot05$ $\delta = 0\cdot40$
Fovea			
$0\cdot00$	$\bar{2}\cdot63$	$\bar{1}\cdot08$	$\bar{2}\cdot39$
$\bar{1}\cdot72$	$\bar{2}\cdot40$	$\bar{2}\cdot94$	$\bar{2}\cdot21$
$\bar{1}\cdot45$	$\bar{2}\cdot22$	$\bar{2}\cdot75$	$\bar{2}\cdot07$
$\bar{1}\cdot11$	$\bar{2}\cdot07$	$\bar{2}\cdot58$	$\bar{3}\cdot89$
Parafovea			
$\bar{1}\cdot99$	$\bar{1}\cdot30$	$\bar{1}\cdot61$	$\bar{2}\cdot82$
$\bar{1}\cdot69$	$\bar{1}\cdot03$	$\bar{1}\cdot38$	$\bar{2}\cdot59$
$\bar{1}\cdot41$	$\bar{2}\cdot77$	$\bar{1}\cdot21$	$\bar{2}\cdot35$
$\bar{1}\cdot07$	$\bar{2}\cdot55$	$\bar{2}$ 92	$\bar{2}\cdot14$
$\bar{4}\cdot40$	—	—	$\bar{4}\cdot54$
$\bar{5}\cdot97$	$\bar{4}\cdot77$	$\bar{3}\cdot51$	$\bar{4}\cdot45$
$\bar{5}\cdot67$	$\bar{4}\cdot65$	$\bar{3}\cdot39$	$\bar{4}\cdot28$
$\bar{5}\cdot36$	$\bar{4}\cdot49$	$\bar{3}\cdot28$	$\bar{4}\cdot14$
$\bar{5}\cdot06$	$\bar{4}\cdot41$	$\bar{3}\cdot18$	$\bar{5}\cdot96$

Subject F. W. C.

$\log_{10} B$	Test spot: $p = 0\cdot75$ $\delta = 0\cdot11$	$p = 0\cdot05$ $\delta = 0\cdot11$	$p = 0\cdot05$ $\delta = 0\cdot40$
Fovea			
$0\cdot01$	$\bar{2}\cdot68$	$\bar{1}\cdot32$	$\bar{2}\cdot66$
$\bar{1}\cdot74$	$\bar{2}\cdot52$	$\bar{1}\cdot17$	$\bar{2}\cdot47$
$\bar{1}\cdot46$	$\bar{2}\cdot37$	$\bar{1}\cdot00$	$\bar{2}\cdot24$
$\bar{1}\cdot12$	$\bar{2}\cdot14$	$\bar{2}\cdot80$	$\bar{2}\cdot02$
Parafovea			
$\bar{1}\cdot99$	$\bar{1}\cdot22$	$\bar{1}\cdot58$	$\bar{2}\cdot95$
$\bar{1}\cdot69$	$\bar{2}\cdot97$	$\bar{1}\cdot39$	$\bar{2}\cdot71$
$\bar{1}\cdot41$	$\bar{2}\cdot77$	$\bar{1}\cdot23$	$\bar{2}\cdot46$
$\bar{1}\cdot07$	$\bar{2}\cdot54$	$\bar{2}\cdot94$	$\bar{2}\cdot21$
$\bar{4}\cdot40$	$\bar{3}\cdot19$	$\bar{3}\cdot98$	$\bar{4}\cdot86$
$\bar{5}\cdot97$	$\bar{3}\cdot21$	$\bar{3}\cdot72$	$\bar{4}\cdot67$
$\bar{5}\cdot67$	$\bar{4}\cdot95$	$\bar{3}\cdot60$	$\bar{4}\cdot43$
$\bar{5}\cdot36$	$\bar{4}\cdot77$	$\bar{3}\cdot42$	$\bar{4}\cdot28$
$\bar{5}\cdot06$	$\bar{4}\cdot70$	$\bar{3}\cdot24$	$\bar{4}\cdot14$

Table VI—Values of $\log_{10}$ T. Conditioning Stimulation: Uniform Surround Brightness B_S. Dark centre field of diameter 1°

Subject B. H. C.

$\log_{10} B_S$	$p = 0·75$ $\delta = 0·11$	$p = 0·05$ $\delta = 0·11$	$p = 0·05$ $\delta = 0·40$
Fovea			
1·20	$\bar{2}$·73	$\bar{1}$·29	$\bar{2}$·69
0·91	$\bar{2}$·58	$\bar{1}$·15	$\bar{2}$·51
0·51	$\bar{2}$·33	$\bar{1}$·00	$\bar{2}$·26
0·26	$\bar{2}$·21	$\bar{2}$·81	$\bar{2}$·11
Parafovea			
1·18	$\bar{1}$·38	$\bar{1}$·77	$\bar{1}$·08
0·89	$\bar{1}$·20	$\bar{1}$·50	$\bar{2}$·87
0·47	$\bar{2}$·87	$\bar{1}$·18	$\bar{2}$·46
0·20	$\bar{2}$·68	$\bar{1}$·04	$\bar{2}$·32
....			
$\bar{5}$·97	$\bar{3}$·49	$\bar{2}$·07	$\bar{4}$·98
$\bar{5}$·67	$\bar{3}$·28	$\bar{3}$·88	$\bar{4}$·80
$\bar{5}$·36	$\bar{3}$·16	$\bar{3}$·77	$\bar{4}$·61
$\bar{5}$·06	$\bar{4}$·89	$\bar{3}$·60	$\bar{4}$·48

Subject W. S. S.

$\log_{10} B_S$	$p = 0·75$ $\delta = 0·11$	$p = 0·05$ $\delta = 0·11$	$p = 0·05$ $\delta = 0·40$
Fovea			
1·20	$\bar{2}$·69	$\bar{1}$·23	$\bar{2}$·57
0·91	$\bar{2}$·51	$\bar{1}$·06	$\bar{2}$·40
0·51	$\bar{2}$·25	$\bar{2}$·82	$\bar{2}$·12
0·26	$\bar{2}$·05	$\bar{2}$·67	$\bar{3}$·97
Parafovea			
1·18	$\bar{1}$·43	$\bar{1}$·60	$\bar{1}$·07
0·89	$\bar{1}$·14	$\bar{1}$·33	$\bar{2}$·78
0·47	$\bar{2}$·79	$\bar{2}$·96	$\bar{2}$·44
0·20	$\bar{2}$·69	$\bar{2}$·91	$\bar{2}$·22
......			
$\bar{4}$·70	$\bar{4}$·81	$\bar{3}$·49	$\bar{4}$·55
$\bar{4}$·40	$\bar{4}$·65	$\bar{3}$·36	$\bar{4}$·39
$\bar{5}$·97	$\bar{4}$·64	$\bar{3}$·38	$\bar{4}$·36
$\bar{5}$·67	$\bar{4}$·50	$\bar{3}$·30	$\bar{4}$·27
$\bar{5}$·36	$\bar{4}$·48	$\bar{3}$·33	$\bar{4}$·23

Subject F. W. C.

$\log_{10} B_S$	$p = 0·75$ $\delta = 0·11$	$p = 0·05$ $\delta = 0·11$	$p = 0·05$ $\delta = 0·11$
Fovea			
1·20	$\bar{2}$·73	$\bar{1}$·32	$\bar{2}$·75
0·91	$\bar{2}$·60	$\bar{1}$·12	$\bar{2}$·49
0·51	$\bar{2}$·37	$\bar{2}$·90	$\bar{2}$·25
0·26	$\bar{2}$·18	$\bar{2}$·77	$\bar{2}$·11

Table VI—(continued)

Subject F. W. C.—(continued)

$\log_{10} B_S$	$p = 0\cdot75$ $\delta = 0\cdot11$	$p = 0\cdot05$ $\delta = 0\cdot11$	$p = 0\cdot05$ $\delta = 0\cdot11$
Parafovea			
$1\cdot18$	$\bar{1}\cdot52$	$\bar{1}\cdot72$	$\bar{1}\cdot25$
$0\cdot89$	$\bar{1}\cdot25$	$\bar{1}\cdot51$	$\bar{2}\cdot97$
$0\cdot47$	$\bar{2}\cdot93$	$\bar{1}\cdot17$	$\bar{2}\cdot62$
$0\cdot20$	$\bar{2}\cdot62$	$\bar{2}\cdot99$	$\bar{2}\cdot39$
$\bar{4}\cdot70$	$\bar{3}\cdot08$	$\bar{3}\cdot67$	$\bar{4}\cdot75$
$\bar{4}\cdot40$	$\bar{4}\cdot91$	$\bar{3}\cdot66$	$\bar{4}\cdot53$
$\bar{5}\cdot97$	$\bar{4}\cdot70$	$\bar{3}\cdot50$	$\bar{4}\cdot45$
$\bar{5}\cdot67$	$\bar{4}\cdot71$	$\bar{3}\cdot44$	$\bar{4}\cdot34$
$\bar{5}\cdot36$	$\bar{4}\cdot68$	$\bar{3}\cdot37$	$\bar{4}\cdot25$
$\bar{5}\cdot06$	$\bar{4}\cdot64$	$\bar{3}\cdot21$	$\bar{4}\cdot14$

Table VII—Values of $\log_{10} T$. Conditioning stimulation: Glare source of angular diameter $0\cdot8°$, placed $6°$ above test spot and producing an eye illumination of E ft. candles. Background brightness zero

		$p = 0\cdot75$ $\delta = 0\cdot11$	$p = 0\cdot05$ $\delta = 0\cdot11$	$p = 0\cdot05$ $\delta = 0\cdot40$
Fovea				
	B. H. C.	$\bar{2}\cdot74$	$\bar{1}\cdot31$	$\bar{2}\cdot44$
$\log_{10} E = 0\cdot36$	W. S. S.	$\bar{2}\cdot61$	$\bar{1}\cdot12$	$\bar{2}\cdot31$
	F. W. C.	$\bar{2}\cdot60$	$\bar{1}\cdot16$	$\bar{2}\cdot45$
Parafovea				
	B. H. C.	$\bar{1}\cdot03$	$\bar{1}\cdot49$	$\bar{2}\cdot73$
$\log_{10} E = 0\cdot36$	W. S. S.	$\bar{2}\cdot88$	$\bar{1}\cdot30$	$\bar{2}\cdot55$
	F. W. C.	$\bar{2}\cdot90$	$\bar{1}\cdot38$	$\bar{2}\cdot65$
	B. H. C.	$\bar{3}\cdot20$	$\bar{3}\cdot76$	$\bar{4}\cdot60$
$\log_{10} E = \bar{4}\cdot01$	W. S. S.	$\bar{4}\cdot63$	$\bar{3}\cdot32$	$\bar{4}\cdot23$
	F. W. C.	$\bar{4}\cdot95$	$\bar{3}\cdot55$	$\bar{4}\cdot39$

The dotted lines joining the high and low brightness ranges are of course hypothetical. They were sketched in having regard to the complete curves already obtained. No foveal measurements were made for the low brightness range as the foveal l.b.i. is constant in that region.

The results for the three subjects are of much the same character except for the range of low brightness surrounds (parafoveal vision). Here, for subject B. H. C. the surrounds and background curves run parallel, the l.b.i. for the surrounds being slightly greater than the l.b.i. for the backgrounds. For subjects W. S. S. and F. W. C., however, the surround l.b.i. values are in general lower than the background values, the two curves intersecting at an appreciable angle. This difference between the subjects is associated with extreme difficulty on the part of F. W. C. and W. S. S. in obtaining any consistent readings with a low brightness surround owing to the erratic fading in and out of the dark centre field which is, of course, seen parafoveally (disappearance of objects viewed parafoveally, on prolonged fixation). For B. H. C. whose parafoveal

l.b.i. values were always found to be higher than those for W. S. S. or F. W. C., this difficulty was much less marked, the dark centre field as a rule being invisible. The explanation of these effects may be that when the dark centre field is invisible to the subject so that the surround field becomes apparently a uniform background the l.b.i. assumes a value appropriate to the uniform

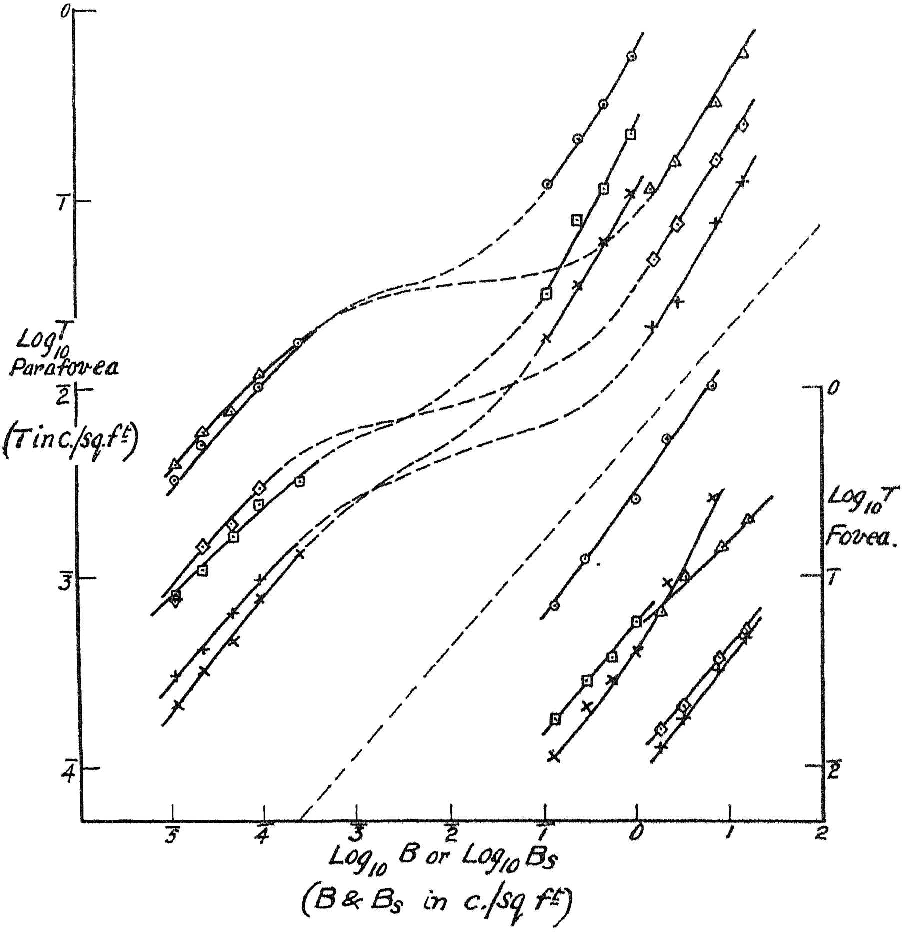

FIG. 21—Subject B. H. C. × Background, 0·4°, 0·05 *s*; ⊙ background 0·1°, 0·05 *s*; ⊡ background 0·1°, 0·75 *s*; + surround 0·4°, 0·05 *s*; △ surround 0·1°, ·05 *s*; ◇ surround 0·1°, 0·75 *s*.

background. If by an eye motion or by blinking visibility of the dark centre field is restored, the l.b.i. immediately assumes a lower value corresponding to the actual surround brightness condition.

A comparison of the values of $\log_{10} T$ obtained with a given test spot, employing uniform background brightness B or surround brightness B_S,

brings out the point that at high brightness levels B_S must be of the order 10 times B to give the same l.b.i. value. This applies to both foveal and parafoveal vision. At low brightness levels (parafoveal vision), for B. H. C., B and B_S must have about the same value to give the same l.b.i. For W. S. S. and

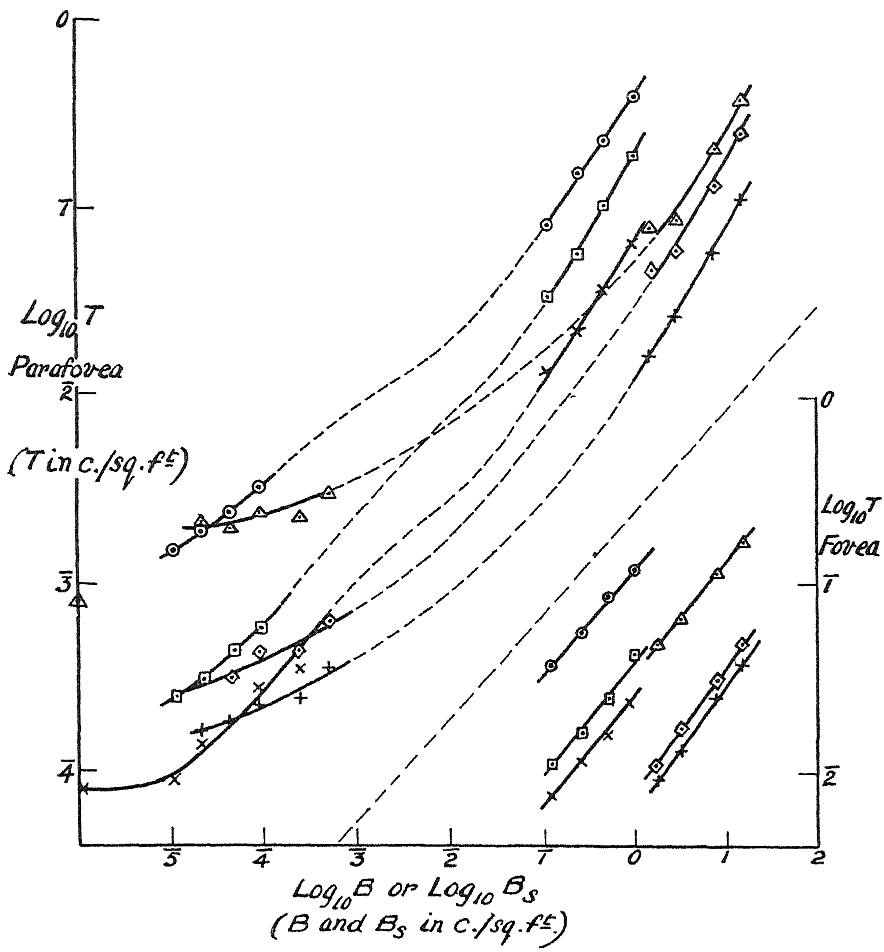

Fig. 22—Subject W. S. S. Key as in fig. 21.

F.W.C. under these conditions, the relation between B and B_S to give the same l.b.i. is vague owing to the effects just described.

In each brightness range and for either surrounds or backgrounds, the l.b.i. data for different test spots lie on approximately parallel lines. The distances apart of these lines measured on the log T axis give information as to the effect of test spot size and exposure time, on the value of the l.b.i.

Confining attention to circular white test spots of angular diameter δ and exposure time p, we may say that if the condition of a retinal area is fixed, *i.e.*, if the conditioning stimulation is fixed, then the l.b.i. T is a function of δ and p. If this function were of the form

$$p \times \delta^2 \times \mathrm{T} = \text{const} = c, \tag{2}$$

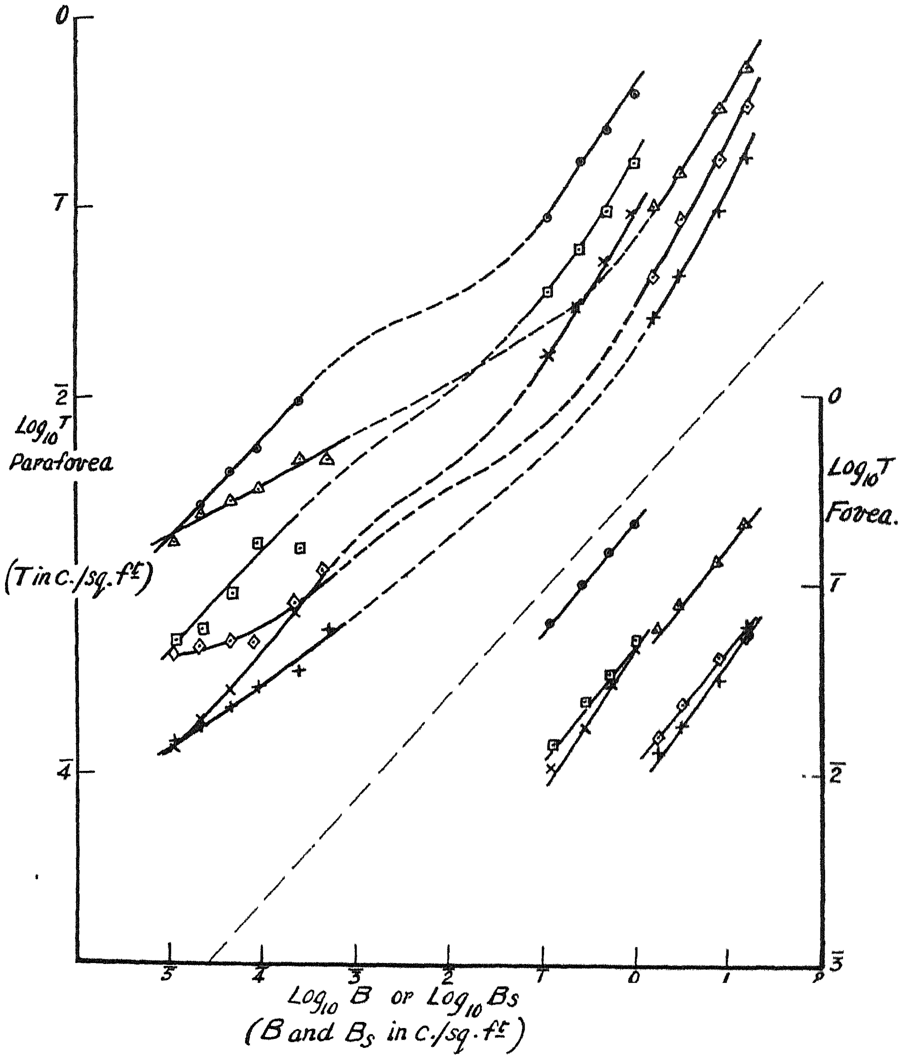

FIG. 23—Subject F. W. C. Key as in fig. 21.

where the constant c is determined by the conditioning stimulation, it would be true to say there is complete spatial and temporal integration. In practice there is complete integration only if p and δ are below certain limits whose

values depend on the conditioning stimulation and on the region of the retina concerned. We wish to find a way of expressing the degree of integration when p and δ exceed the limits mentioned.

We shall assume that for test spot sizes and flash periods in the neighbourhood of any particular pair of values of δ and p, the relation of T to δ and p is approximately of the form:

$$\mathrm{T} \times p^{r} \times (\delta^2)^{s} = \text{const.} = c. \tag{3}$$

If r or s equals unity, temporal or spatial integration is complete, whereas, if r or s equals zero, temporal or spatial integration is absent.

Taking logarithms of (3) we have

$$\log \mathrm{T} = \log c - r \log p - 2s \log \delta. \tag{4}$$

If two test spots with the same exposure time p, but of different diameters δ_1 and δ_2 lead to the l.b.i. value $\log \mathrm{T}_1$ and $\log \mathrm{T}_2$, it follows from (4) that

$$\log \mathrm{T}_1 - \log \mathrm{T}_2 = 2s (\log \delta_2 - \log \delta_1).$$

Taking, for example, fig. 21, we see that for parafoveal vision and background brightness stimulation in the range $\log \mathrm{B} = \bar{1}\cdot 07$ to $\bar{2}\cdot 00$ we have two sections of curve one corresponding to $p = 0\cdot 05$, $\delta = 0\cdot 4^\circ = \delta_1$, the other to $p = 0\cdot 05$, $\delta = 0\cdot 11^\circ = \delta_2$. The curves are approximately parallel in the range considered and correspond to a value of $\log \mathrm{T}_1 - \log \mathrm{T}_2$ equal to $\bar{1}\cdot 25$. Thus, for this case,

$$s = \frac{\bar{1}\cdot 25}{2 \log (0\cdot 11/0\cdot 4)} = 0\cdot 66.$$

In Table VIII are given the values of s deduced in this way, for different conditioning stimulations. The results for the different subjects lead to much the same s values and the mean data for all three subjects have therefore been used in deriving the figures given in the table. It will be observed that in addition to background and surround brightness conditioning stimulations, results for stimulation by a glare source are also included.

An examination of Table VIII shows that for any conditioning stimulation corresponding to a high brightness level, s has a value in the neighbourhood of $0\cdot 64$. For the low brightness levels and for parafoveal vision, on the other hand, s is approximately unity, *i.e.*, integration is complete.

The index of temporal integration r is arrived at by a precisely similar method and the values derived are shown in Table IX. It appears that for a test spot of $0\cdot 11^\circ$ diameter temporal integration as measured by r is greatest

Table VIII—Values of s (index of spatial integration) derived from l.b.i. values at $\delta_1 = 0\cdot 4°$, $\delta_2 = 0\cdot 11°$

Brightness levels	Conditioning stimulation	Foveal vision $p = 0\cdot 05$	Parafoveal vision $p = 0\cdot 05$
High	Background brightness in the range $\log B = \bar{1}\cdot 0$ to $0\cdot 0$	$0\cdot 65$	$0\cdot 67$
	Surround brightness in the range $\log B_S = 0\cdot 20$ to $1\cdot 20$	$0\cdot 59$	$0\cdot 51$
	Glare: $\log E = 0\cdot 36$. Source 6° above test spot.	$0\cdot 72$	$0\cdot 68$
Low	Background brightness in the range $\log B = \bar{5}\cdot 0$ to $\bar{4}\cdot 4$		$1\cdot 02$
	Surround brightness in the range $\log B_S = \bar{5}\cdot 0$ to $\bar{4}\cdot 4$		$0\cdot 95$
	Glare: $\log E = \bar{4}\cdot 0$. Source 6° above test spot		$1\cdot 02$

Table IX—Values of r (index of temporal integration) derived from the l.b.i. values at $p_1 = 0\cdot 05$ sec. and $p_2 = 0\cdot 75$ sec.

Brightness level	Conditioning stimulation	Foveal vision $\delta = 0\cdot 11°$	Parafoveal vision $\delta = 0\cdot 11°$
High	Background brightness in the range $\log B = \bar{1}\cdot 0$ to $0\cdot 0$	$0\cdot 50$	$0\cdot 36$
	Surround brightness in the range $\log B_S = 0\cdot 20$ to $1\cdot 20$	$0\cdot 49$	$0\cdot 23$
	Glare: $\log E = 0\cdot 36$. Source 6° above test spot	$0\cdot 47$	$0\cdot 38$
Low	Background brightness in the range $\log B = \bar{5}\cdot 0$ to $\bar{4}\cdot 4$	—	$0\cdot 56$
	Surround brightness in the range $\log B_S = \bar{5}\cdot 0$ to $\bar{4}\cdot 4$	—	$0\cdot 58$
	Glare: $\log E = \bar{4}\cdot 0$. Source 6° above test spot	—	$0\cdot 55$

in the parafovea at low brightness levels, rather less for the fovea at high brightness levels and least for the parafovea at high brightness levels.

7—*Coloured Conditioning Stimulation*

In this section will be given the results obtained using a coloured conditioning stimulation. All the measurements were made with apparatus II and using monocular vision. The test spot employed throughout was rectangular in form, its horizontal and vertical sides subtending respectively angles $0\cdot 70°$ and $0\cdot 16°$ at the subject's eye. The conditioning stimulation was either a surround brightness with a circular centre dark field of $1\cdot 28°$ angular diameter, or a small glare source situated 3° vertically above the test spot. The glare source was the image of the incandescent square tungsten plate of a pointolite, the side of the square subtending an angle of $0\cdot 8°$ at the subject's eye. To obtain conditioning stimulations of various colours the liquid spectrum filters

mentioned in Section 2 were inserted in the glare source beam or in the beams used to produce the surround brightness. The intensity of the glare source with the colour filter in position was determined by the illumination produced on a vertical plane at the position of the subject's eye. An opal diffusing glass could be set up in this position and its brightness determined by viewing it with the flicker illuminometer through an aperture Z in the wooden cube (see fig. 2). Owing to the low transmissions of some of the colour filters the brightness of the opal was too low to obtain a satisfactory measurement in this way. In such cases the transmission of the colour filter for the light of the pointolite was measured by placing the opal quite close to the glare source and taking flicker illuminometer readings of its brightness with and without the colour filter in position. Knowing the transmission of the colour filter and the white light illumination in the eye plane, the maximum coloured light illumination could be determined. The lower intensities of the glare source were obtained by inserting in the glare beam either photographic filters which can be assumed approximately neutral, or " neutral " glass filters whose transmissions for the different colours used were determined directly.

A general exploration of the whole field of study was first carried out. For each of the two subjects (B. H. C. and W. S. S.) runs were made to determine the effect on the l.b.i. of steadily increasing the glare source intensity or the surround brightness. Before any run, the subject was kept in darkness for an hour. Actually during this hour readings were taken of the 5°-parafoveal l.b.i., but these observations need not concern us here. At the expiration of the hour a glare source or surround brightness of a particular colour was switched on at the lowest intensity to be studied, and the main readings were commenced.

The results for the glare source are reproduced in figs. 24–27 where log T is plotted against log E.

It is clear that for foveal vision the same type of variation of log T is obtained for all three colours tested. There is no well-defined effect on log T until $\log_{10} E$ exceeds $\bar{3}$ and thereafter the curves all rise in more or less similar fashion. The results are consistent with the further measurements of the effect of glare of different colours on foveal vision for white, which will be given below and which show that the glare effect is to a first approximation independent of the colour of the glare source.

Turning now to the effect of glare on 5°-parafoveal vision, we see that the curves tend to converge at the higher values of $\log_{10} E$ (above about $\bar{3}$), but below this value the curves for the different coloured glare sources move apart

and then finally converge when the condition of total darkness (zero glare intensity) is reached. The conclusion to which these observations point is that if the relative intensities of different coloured glare sources are so fixed that

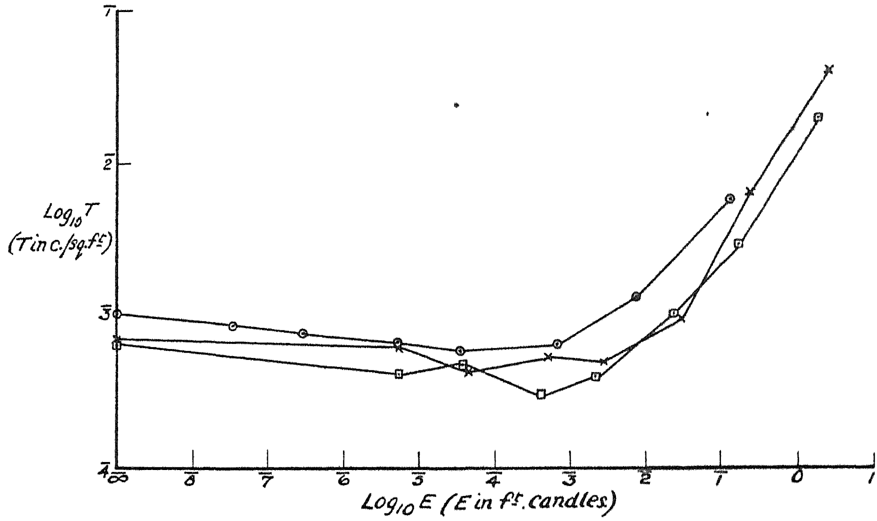

FIG. 24—Subject B. H. C. ⊙ blue (filter A′); ⊡ green (filter C); × pale red (filter E).

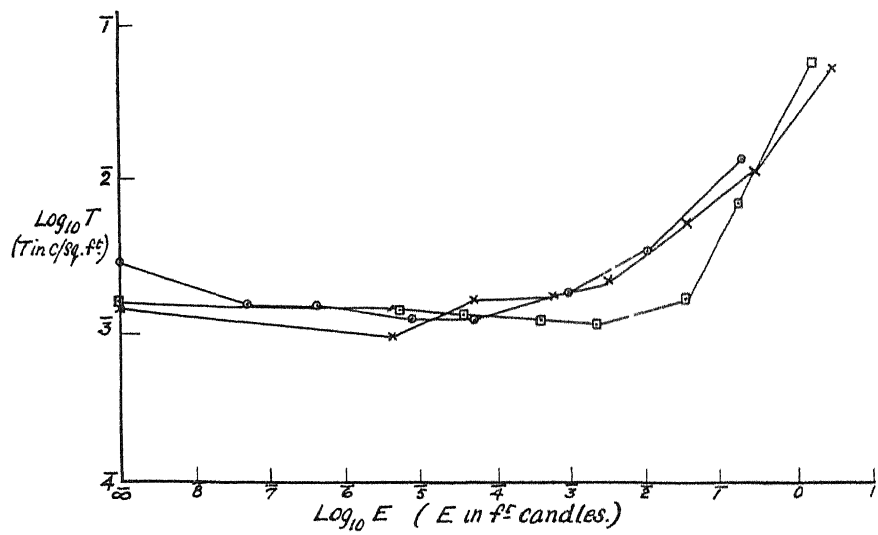

FIG. 25—Subject W. S. S. ⊙ blue (filter A′); ⊡ green (filter C); × pale red (filter E).

Figs. 24, 25—Foveal vision. Glare source of colour A′, C, or E.

they raise the 5°-parafoveal l.b.i. for white to the same extent when the absolute intensity is sufficiently high ($\log_{10} E > \bar{3}$ for a glare source 3° above the test spot), then for very low absolute intensities, the 5°-parafoveal l.b.i. is raised

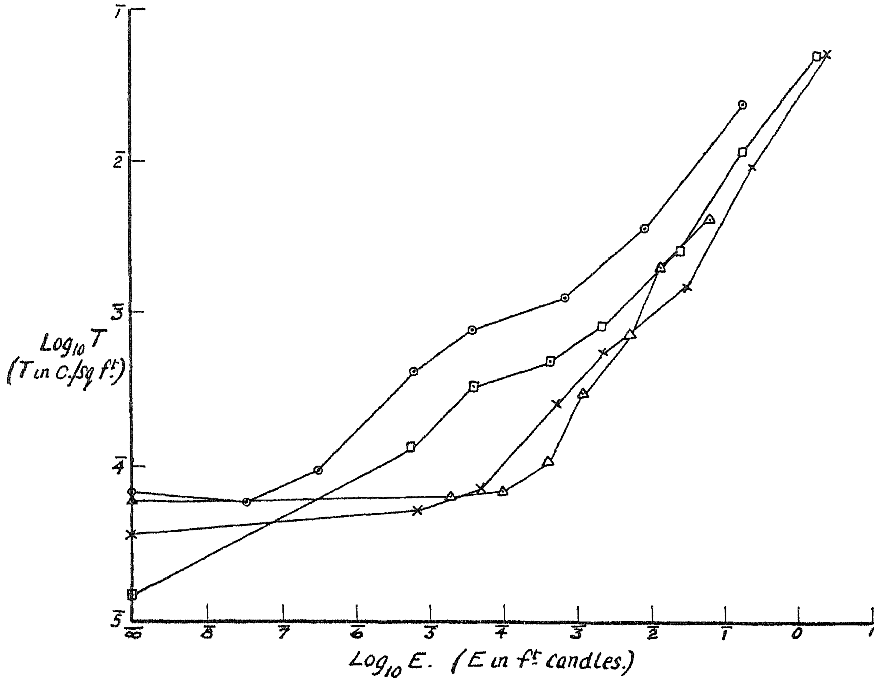

FIG. 26—Subject B. H. C. ⊙ blue (filter A′); ⊡ green (filter C); × pale red (filter E); △ red (filter F).

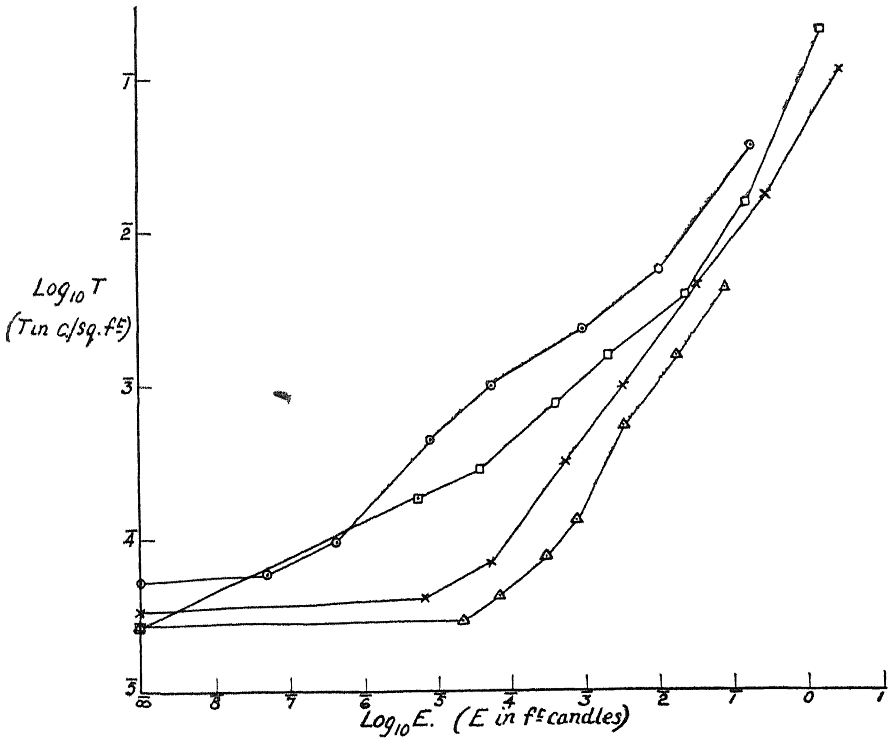

FIG. 27—Subject W. S. S. ⊙ blue (filter A′); ⊡ green (filter C); × pale red (filter E); △ red (filter F).

most by the blue glare source and least by the red. In other words, the glare-producing efficiency of a light source exhibits a type of Purkinje phenomenon. It should be noted that the glare source brightness is itself relatively high in the region where the l.b.i. curves exhibit this Purkinje effect. Thus when $\log_{10} E = \bar{4}$ the brightness of the glare source equals approximately 0·5 candles/sq. ft.

The data for the effect of different coloured surrounds on the l.b.i. for white are more erratic than the corresponding results for coloured glare sources, but are of the same general character. As far as foveal vision is concerned, a later series of measurements indicates that surrounds of different colours produce the same effect on the l.b.i. just as in the case of a glare source.

A special study was then made of the effect of glare sources of different colours on foveal vision for white. It was found that for a given glare illumination at the eye the value of the l.b.i. is independent of the colour of the glare source as far as the precision of the measurements enables us to judge. As no comparison had been made with a white glare source, this work was repeated at a later date introducing a white glare source and using the multiple decision method. Fig. 28 shows the results of a single run for each glare colour and for white. The two points plotted for each colour at each glare intensity represent the appearance and disappearance l.b.i. values. Using the multiple decision method the appearance and disappearance on l.b.i.'s show no systematic difference and represent in effect independent determinations. The results confirm the conclusion that different coloured glare sources including white have the same effect on the foveal l.b.i. for white, provided they produce the same illumination at the subject's eye.

In all the above measurements no lens or other optical device was interposed between the glare source and test spot and the subject's eye. This avoided any difficulty due to parasitic scattering or interreflection of light into the subject's eye. On the other hand, as the test spot was formed on a surface only 23·6 inches in front of the subject's eye, it was necessary for the subject to accommodate for near vision in order to see the test spot sharply in focus. Some doubt was felt as to whether this accommodation was taking place for all types of conditioning stimulation. The measurements were therefore repeated using an eye lens of 1·75 dioptres which in effect shifts the apparent position of the test spot and the glare source to infinity. Thus, when using the eye lens the subject allowed his accommodation to relax as in distant vision. The lens was kept carefully cleaned when making observations. The results are plotted in fig. 29. As regards the effect of colour of glare source,

the data simply confirm the previous conclusion. Comparing the absolute values of the l.b.i. with those obtained without an eye lens, however, we note that for one subject (B. H. C.) there is practically no difference whereas for the other subject (W. S. S.) the l.b.i. is uniformly lower when using the eye lens. The effect of this lowering of the l.b.i. for W. S. S. is to bring the data for the

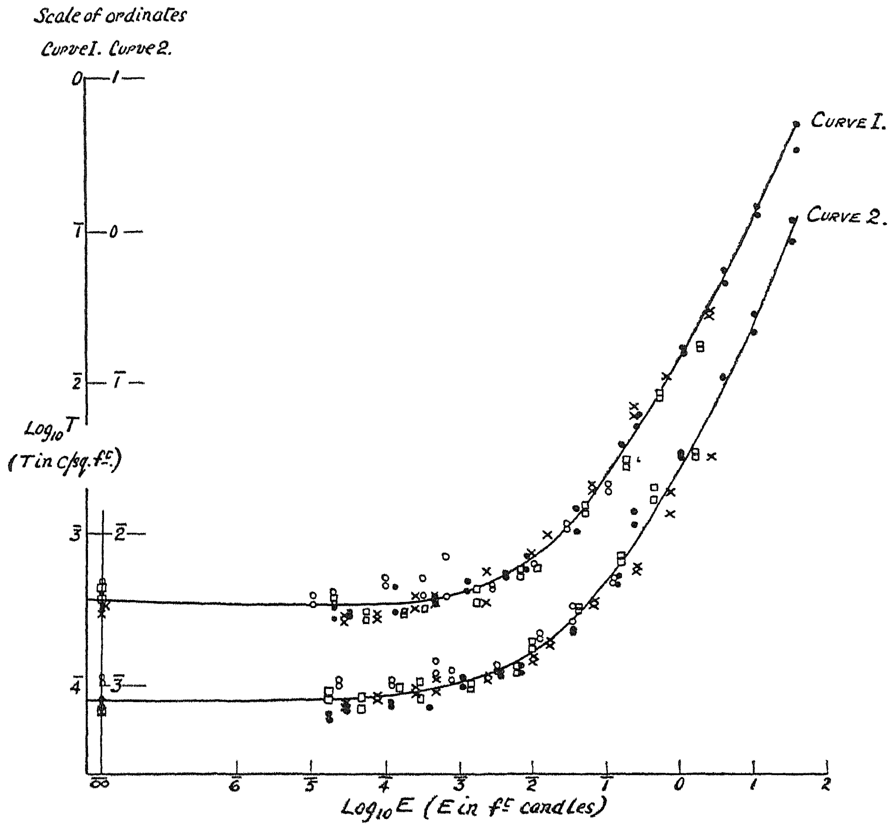

FIG. 28—Curve I, B. H. C.; curve II, W. S. S.

○ blue (filter A′); □ green (filter C); × pale red (filter E); ● white.

two subjects much closer together. We may perhaps conclude that accommodation to near vision failed to occur for subject W. S. S. It should be noted that in the earlier measurements an attempt was made to aid accommodation by projecting on to the background screen S two weak points of light one on either side of the test spot and separated from it by $2\frac{1}{2}°$.

The results of a further study for the subject B. H. C. of the effect of different coloured surrounds on the foveal l.b.i. for white are shown in fig. 30. The

measurements fail to show any marked variation of the l.b.i. with colour of the surround for a given brightness of the latter. This result fits in with our conclusions on the effects of glare sources of different colours on foveal vision for white.

For subject W. S. S. only one run with a coloured surround was made (apart from the preliminary runs), but the results (shown in fig. 21) are of interest

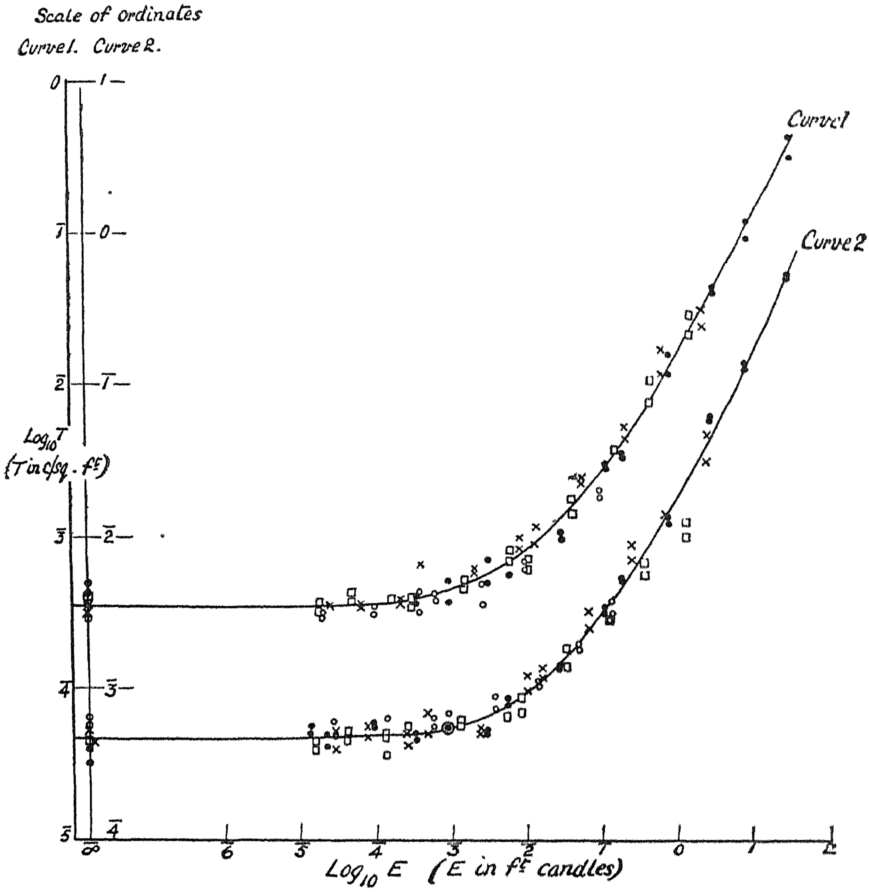

FIG. 29—Curve 1, B. H. C.; curve 2, W. S. S.

○ blue (filter A′); □ green (filter C); × pale red (filter E); ● white.

because they bear on the question of accommodation. The measurements were made without an eye lens, and it is seen that as the surround brightness decreases log T drops steadily and attains a lowest value of the order of $\bar{4}\cdot 7$ which is very near to the minimum value of log T in the glare experiments using an eye lens (fig. 29). When the surround brightness gets very low,

however, log T rises to a value of the order $\bar{3}\cdot 0$ and this approximates to the minimum value of log T in the glare experiments without an eye lens (fig. 28). This behaviour of log T is consistent with the view that at very low brightness levels in the field, accommodation for near vision did not occur, but as the surround brightness increased (showing more clearly the outer frame and the edge of the circular centre field) accommodation became operative. In the measurements with the glare source using no eye lens apparently accommodation was not obtained for W. S. S. even at high glare intensities.

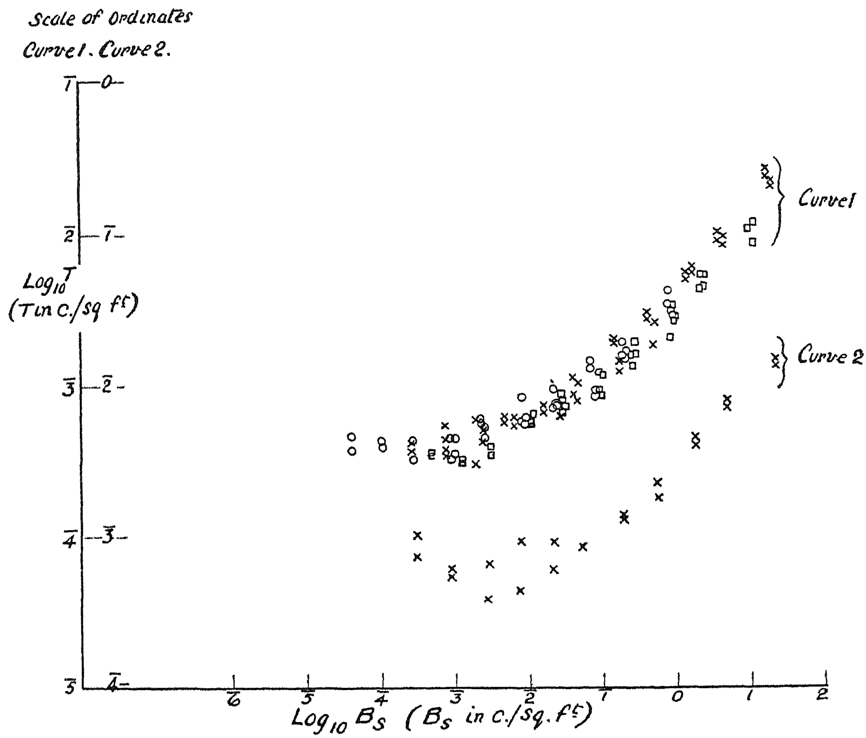

FIG. 30—Curve 1, B. H. C.; curve 2, W. S. S.
○ blue (filter A′); □ green (filter C); × pale red (filter E).

8—*Conclusion*

The main points dealt with in this paper may be summarized here.

(1) Estimates have been made of the order of magnitude of the two types of error to which measurements of the l.b.i. are subject, namely, chance error and day to day variations. It appears that a redetermination of the l.b.i. differs from the original value on the average by 20% when the stimulation

conditions are as near as possible identical. Such deviations are much larger than can be accounted for by the indefiniteness of the l.b.i. value at any one time.

(2) Data are given for the variation of the foveal l.b.i. with the brightness of a uniform background or uniform surround. Above a certain brightness the Fechner fraction is approximately constant. A background brightness is about 10 times as effective as a surround brightness in raising the foveal l.b.i. to a given value.

(3) Curves are obtained showing the variation of the l.b.i. for 5°-parafoveal vision with the intensity of a white conditioning stimulation. For all types of test spot and conditioning stimulation the curves exhibit a characteristic division into two ranges, a high intensity range associated with cone vision and a low intensity range associated with rod vision.

(4) Data for different test spot diameters and flash periods are used to determine indices of spatial and temporal integration appropriate to foveal and parafoveal vision at high and low brightness levels.

(5) It is shown that to a first approximation the foveal l.b.i. for white is independent of the colour of the conditioning stimulation (surround or glare source) and is determined by the intensity of the latter.

(6) For parafoveal vision coloured conditioning stimulations exhibit a type of Purkinje effect. For a glare source conditioning stimulation the effect occurs in a range of glare source intensities above the value at which the glare source brightness itself would show the Purkinje effect.

The authors have pleasure in thanking Mr. F. W. Cuckow, B.Sc., for assistance in some of the measurements. The work was carried out under the auspices of the Illumination Research Committee of the Department of Scientific and Industrial Research.

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An Experimental Investigation of the Measurability of Auditory Sensation

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(Communicated by T. Graham Brown, F.R.S.—Received May 12, 1934)

The conception of a "scale of sensation" has been the centre of a considerable amount of discussion in psychological literature. Confining attention to the question of intensity it has been admitted that the formulation of a scale relating sensation-intensity to stimulus-intensity may be a matter of difficulty and doubt even for one particular sense, and for different senses different relations may hold; but it has been commonly supposed that theoretically there must be some such relations. In other words a particular strength of sensation must be uniquely associated with a particular stimulus, and this sensation may be measured, that is, assigned a quantitative value on a scale, which may be empirical, but is none the less a scale of magnitude of sensation.

This assumption has been questioned (Myers, 1931) usually from the theoretical side; it is pointed out that more or less unverifiable or at best unverified, assumptions are made in the formulation of such supposed scales. Thus in the case of sound the decibel is often regarded as a measure of loudness-sensation, but this has been criticized not only on the experimental ground that the scale of decibels may not in fact run parallel with the true scale of loudnesses, but that it is actually only a scale of stimuli and not a measure of sensation at all.

It appears therefore desirable to test experimentally in a definite case whether a point to point correspondence can be made out between intensities of sensation and the intensities of the corresponding stimuli which produce those sensations. The present work concerns the question of loudness in the sense of hearing. In the first place we must decide what sensation judgments are to be made. In the supposed scale of the Weber-Fechner equation it is assumed that just perceptible differences of sensation represent equal steps of sensation, that is successive divisions in a sensation scale. Other methods of approaching the problem use larger sensation differences. One such means is

to attempt to "bisect" a given interval between two sensations of different strengths, that is in sound of given pitch, to find a note of intensity B such that it appears to lie half-way in loudness between two other notes of intensities A and C respectively, the sounds A, B, and C being applied to the same ear in succession.

This is the method adopted in the present work. It was first ascertained by testing a large number of subjects that the problem was one capable of being definitely answered. All agreed that they could find such a half-way loudness with more or less precision. The subjects differed greatly in their understanding of the problem, but it was not found that this had any effect on their satisfaction that bisection is possible.

What precisely the "bisection" implies is not clear, whether for instance the judgment is that (loudness of A — loudness of B) = (loudness of B — loudness of C) or that the ratio of the loudness of A to the loudness of B bears the same relation as the loudness of B is to the loudness of C, or any other relation, but all agree that such a note B can be found and that it does satisfy the ear as lying evenly (as regard loudness) between A and C.

The complete experimental procedure adopted was as follows. (1) To bisect (in the sense stated above) the loudness interval between two notes of the same frequency. (2) and (3) To bisect again the two intervals thus obtained, thus getting two fresh loudnesses "$\frac{1}{4}$-way" and "$\frac{3}{4}$-way" between the original notes. (4) Finally to bisect the interval between these "$\frac{1}{4}$-way" and "$\frac{3}{4}$-way" notes. Then the final bisection should reproduce the original "$\frac{1}{2}$-way" note in loudness, that is, should be given by a stimulus of the same intensity as the original "bisecting" loudness; if there is no such agreement, and if the discrepancy is significant, *i.e.*, well outside the overlapping of the probable errors of the experiment, it can only be concluded that loudness intervals as "measured" in this way cannot be correlated definitely with measurements of intensities of stimuli. Another way of stating this is to say that a sensation-scale founded on such intervals of loudness does not exist. And if such a scale fails it follows that other scales (based for instance on the idea of equality of just-perceptible-differences) must also fail. Equal numbers of such supposed small steps need not necessarily correspond with equal intervals in the "scale" under present consideration. Thus it might be possible to correlate the five points obtained in our work (the two original points and the points found in the first, second, and third bisections) with five points on the just-perceptible-difference scale, but both scales would break down together when the test of the final bisection is applied.

In this type of investigation the question arises as to the number of observers to use, whether it is better to take a large number of relatively untrained subjects and apply statistical methods to the results obtained, or to confine the work to a small number of trained observers and make more precise and extensive investigations of each individual. The first method mixes good and poor observers and may lead to small but significant differences being masked by the totality of results. After having made a large number of accurate observations on two right ears we found that the results were of sufficient clarity to justify definite conclusions being drawn.

Method

The experimental procedure is therefore as follows. Sensations corresponding to the intensities X_1 and X_5 are bisected by the sensation corresponding to the adjustable intensity X_3. Similarly X_2 bisecting X_1 and X_3 is determined, and X_4 bisecting X_3 and X_5. Then X'_3 bisecting X_2 and X_4 is found and the results are analysed by statistical methods to determine if the difference between X_3 and X'_3 is significant. If the difference is significant, then it is not possible to construct a loudness scale of sensation for sound.

As the intensities used cover an energy range of about 10^7 to 1, they are expressed logarithmically as decibels above the threshold ; it must be emphasized that this is only a way of specifying the physical intensity of the stimulus and is in no way a measure of sensation.

The use of the physiological threshold as a datum line is in some ways not so satisfactory as some purely physical level of intensity such as 1 dyne per sq cm R.M.S. pressure, but it is more convenient since absolute measurements of sound intensity are extremely difficult. It is better to use the threshold as a datum line than some arbitrary physical constant such as 1 volt R.M.S. pressure across the telephone. A control of the physical intensity of the output circuit was obtained from time to time by the use of a cathode ray oscillograph. The physical constancy of the threshold was thus checked but not measured.

The Apparatus

The sound-producing apparatus consists of a telephone receiver, resistance 2000 ohms, driven by the following electrical circuits. A pure tone heterodyne oscillator enables any frequency up to approximately 11,000 cycles per second to be obtained. This oscillator was fitted with diode rectification in place of

the anode-bend rectification originally described (Shaxby and Gage, 1932). This alteration considerably improved the wave-form of the oscillations produced. One stage of voltage amplification is included in the same unit as the oscillating circuits and rectifier, and this is followed by two further stages of orthodox resistance—capacity amplification. The output end of the amplification sequence is shown in fig. 1. The volume control unit comes between the output valve V_4 and the penultimate valve V_3. It consists of the resistances R_A, R_C and the three resistances R_D, R_B and $R_{B'}$. R_A and R_C each have a resistance of 316000 ohms, and have a series of tappings on them arranged to correspond to steps of 5 decibels, making a total of 70 decibels. (The calibra-

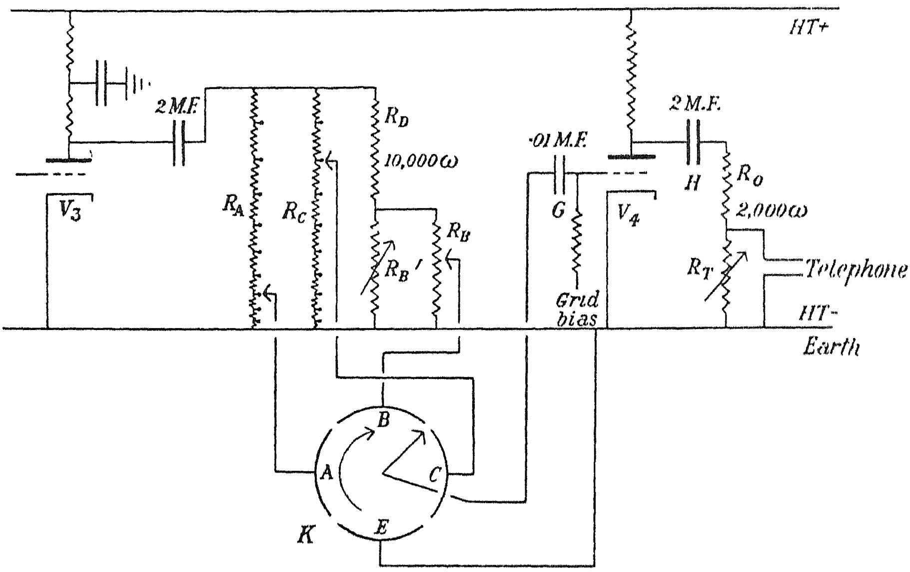


FIG. 1

tion of these "decibel boxes" is described in the appendix to this paper.) R_D is 10000 ohms and $R_{B'}$ consists of a 10000 ohms resistance box variable in steps of 1 ohm. In parallel with $R_{B'}$ is the resistance R_B which is about 50000 ohms and of the commercial wire-wound type used in radio work. The tappings on this resistance enable a range of approximately 18 decibels to be covered in steps which may be considered infinitesimally small; but altering $R_{B'}$ this can be extended to cover a range of 65 decibels.

The tappings on R_A, R_B and R_C must be consecutively connected to the grid of the output valve V_4 through the condenser G by the rotating commutator K, shown diagrammatically in fig. 1. The segments A, B, and C correspond to the resistances R_A, R_B, and R_C respectively, and the fourth

segment E earths the rotating arm thus ensuring silence for a length of time equal to one-quarter of a revolution of the commutator.

The slight apparent complication of the condenser G and its associated grid leak is in order to avoid putting the " earth " ends of R_A, R_B and R_C on the grid bias battery of the output valve V_4. This would lead to inaccuracy at the smaller values of intensity, since the internal resistance of the grid bias battery would be added to the calculated resistance. Also difficulty would arise in ensuring complete silence when the commutator is on section E.

The output valve V_4 is of the 150 volt class taking about 15 mA. It is a mistake to use large power valves in this type of apparatus for feeding a telephone; the telephone " overloads " at amplitudes of oscillation which are quite insufficient to cause distortion in the output valve. An advantage of this small power valve is that the final output circuit can be resistance-capacity fed from the anode of the valve without using excessive supply voltages. There is thus no iron core transformer in the whole apparatus. The output network is shown to the right of the valve V_4 in fig. 1. The telephone is connected in parallel with the variable resistance R_T, which is fed from the anode of V_4 through the condenser H and resistance R_0. R_0 is fixed at 2000 ohms, and R_T is adjused to fix the threshold before each series of observations. When this is being done, the commutator K is placed so that R_A is in action and fixed at 0 decibels. (This is the lowest notch on the resistance R_A and must not be confused with the bottom end of the resistance which is connected to earth and is $-\infty$ decibels.)

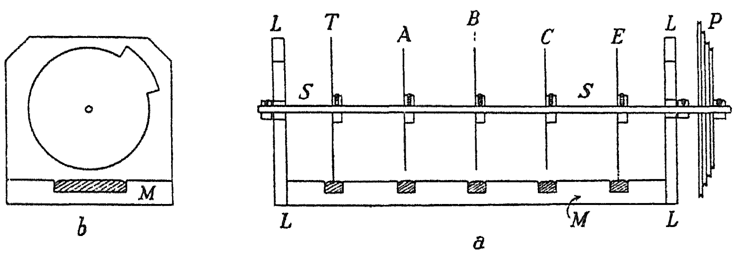


FIG. 2

The mechanical construction of the commutator K is shown in fig. 2. It consists of a series of rotating copper sectors dipping into mercury. The discs are thoroughly amalgamated and very little trouble has been experienced through fouling of the mercury surfaces. The mercury was renewed from time to time and this is probably less trouble than taking more elaborate precautions to keep it clean; the current is very small since it has to pass

through the condenser G, fig. 1, and its associated grid leak. Returning to fig. 2, the shaft S rotates in brass bushes fitted in the ebonite end-pieces LL. This shaft has five copper discs attached to it, each disc dipping into its appropriate mercury trough cut in the ebonite base M. The end disc T is a complete circle and makes contact continuously with the mercury, it is connected to the condenser G, fig. 1. The rotating shaft is thus connected to this condenser. The other discs A, B, C, and E, fig. 2A, are of the shape shown in fig. 2B and the projecting sector is of such an angle that contact is made with the mercury for one-quarter of a revolution. A fine adjustment of the equivalent sector angle can be obtained by adding or removing a little mercury. The discs A, B, C, and E correspond to the similarly lettered sections of the commutator K in fig. 1.

The commutator is rotated by the pulley P driven by a small synchronous motor connected to the A.C. mains through a step-down transformer. This ensures great constancy in the speed of rotation of the commutator. Of more importance is the fact that this type of motor, unlike the ordinary small machine, causes no interference with the valve circuits, a valuable feature when so much of the wiring is not shielded.

The adjustment made by the subject during an experiment is to vary the position of the tapping on R_B, fig. 1 ; the knob of this resistance was therefore removed and replaced by a large pulley wheel of 22 cm diameter, on the circumference of which is inscribed a scale of degrees, fig. 3. A string passes round this pulley, one end of the string entering a sound proof room in which the subject is placed ; the other end is tied to a counter-weight W. In this way

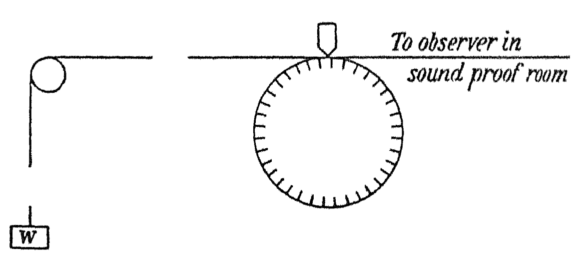


FIG. 3

the subject can vary the tapping on R_B, fig. 1 ; pulling the string to make the sound louder and releasing it to make it less loud. It is important to see that the portion of the string which is in the sound proof room has no reference marks such as knots or worn portions, so that the subject can obtain no indication from the string as to the settings of the resistance R_B.

The sound proof room is supplied with leads for the telephone and also a separate circuit for a tapping key connected to a signal lamp which is outside the room and is visible to the assistant taking the observations. In this way the subject is able to indicate when he considers he has made a " bisection " correctly and desires an observation to be recorded.

The wave form of the note obtained from the oscillator and amplifier was examined by a cathode ray oscillograph of the low voltage type. The alternating potential across R_0 and R_T, fig. 1, is applied to the vertical deflecting plates of the oscillograph through a 5 to 1 step up transformer in order to give a convenient amplitude. The horizontal deflecting plates of the oscillograph are connected to a linear time base sweep circuit " locked " to the vertical oscillations (Watt, Herd and Bell, 1933). A tracing made from a photograph taken with this apparatus is shown in fig. 4. The 5 to 1 transformer will, if anything, make the note less pure; furthermore the maximum amplitude obtainable from the apparatus was used to take the oscillogram. It follows that the note used for the experiments will not be less pure than that shown in fig. 4. The intensity given by the telephone corresponding to this maximum amplitude is about 80 decibels above the threshold for a frequency of 800 and the actual sound obtained is far from pure. This is evident even to the most inexperienced ear. The apparatus is therefore able to overload the telephone before itself giving distortion and the use of the relatively small output valve is justified. During the actual experiments amplitudes as large as these were never used, the maximum being approximately 60 decibels above the threshold. These gave a very satisfactory approximation to a pure note.

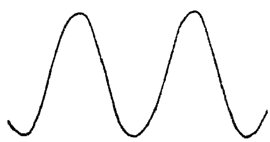


FIG. 4—Frequency = 800

Calibration of the Apparatus

Since this type of apparatus is of very general use for research in physiological acoustics, the methods adopted for calibrating it are of more than immediate interest. Two calibrations are required, (*a*) of the oscillator dial, for frequency, and (*b*) of the network of resistances between the penultimate valve and the output valve, for intensity.

The calibration for frequency is a relatively simple matter if a cathode ray oscillograph is available. Methods using the frequency controlled 50-cycle mains have been described (Watt, Herd and Bell, 1933), which are quite easily applied to this apparatus, more than sufficient amplitude being available for their use.

The calibration for intensity is a much more complicated matter, and must, theoretically, be done for a large number of different frequencies. Since, however, all the present work has been done at a frequency of 800 cycles per second the calibration has only been completely performed for this frequency. It is assumed that the acoustical output of the telephone is proportional to its electrical input, an assumption which is supposed to hold if no overloading takes place; the final conclusion drawn from this work will not depend on its truth. The decibel boxes R_A and R_B must be calibrated for the chosen frequency of 800 cycles per second. The first step is to calibrate the resistance R_B to which is attached the pulley wheel, fig. 3. This resistance will be called for reference the "wheel resistance." Since its calibration is of use in the calculation of the results of the "bisection" experiments it is described here, the calibration of the decibel boxes being given in the appendix.

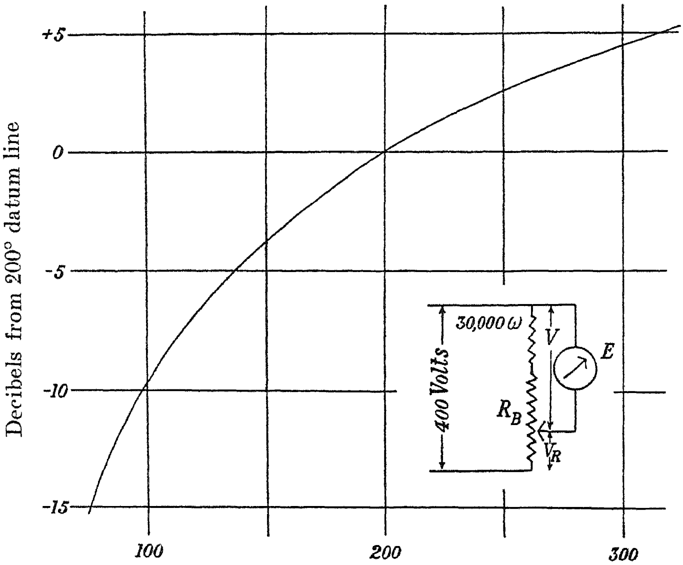
Certain assumptions are made as to the use of the wheel resistance. The value of this unit is about 50000 ohms, and it is taken for granted that the load imposed by the grid circuit of the output valve will have no appreciable shunting effect on the wheel resistance. To be sure of this a resistance of some megohms could be put in series with the grid circuit, but this seemed unnecessary since an error of 1% in the calibration will correspond to an error of 0·086 decibel, much less than can be aurally detected.

The wheel resistance will have distributed capacity and inductance. These are in any case small owing to the type of construction adopted, and as they are evenly distributed along the length of the resistance element the calibration for resistance may be taken to include them.

The wheel resistance is therefore removed temporarily from the amplifier circuit and calibrated using the circuit shown in fig. 5. The reason for the use of this electrostatic voltmeter circuit is that there is a certain amount of resistance at the sliding contact; this is of no consequence in the very high resistance grid circuit in the amplifier but is sufficient to preclude the application of, say, a bridge circuit for calibration. The voltage V for different settings of the wheel is measured by the voltmeter and $V_R = 400\text{-V}$. On plotting $20 \log V_R$ against wheel reading a graph is obtained connecting wheel reading with decibels; the zero is conveniently taken at the arbitrary level when the wheel is at the 200° mark. Fig. 5 shows the resultant curve. This fundamental calibration was performed from time to time during the course of the work to make sure that there was no wear in the wheel resistance. The measurements indicated that any wear taking place is smaller than can be observed.

This method of calibration does not give accurate results at the lower values of the wheel setting, since the corresponding readings for V_R are small and subject to a relatively great inaccuracy. The lower end of the wheel resistance was therefore not used in the further calibration of the apparatus.

The calibration from this point is performed acoustically and not by the use of a valve voltmeter or similar measuring device for alternating currents. The acoustic method is much more sensitive for the lower intensity ranges and it has the further advantage that for high intensities any unwanted oscillations of frequencies other than 800 per second can easily be neglected by the ear.



Wheel reading

Fig. 5

It is well known that a full-wave high tension rectifier supply unit working on 50 cycles A.C. mains will give a certain amount of 100 cycle hum which is difficult to eliminate. This may be quite innocuous acoustically since the sensitivity of the ear is low at 100 cycles per second but a valve voltmeter would be greatly influenced and give a spurious indication.

The Experimental Results

The "bisection" experiments were all made at the same frequency, 800 cycles per second and with the same period of rotation of the commutator, one revolution is 5·034 seconds. This speed was found to be convenient with the apparatus available; it is not fast enough to cause splashing of the mercury

and not too slow to prevent accurate judgment of bisections. Rough tests suggested that the bisection settings may depend on the speed of the commutator, but this point was not pursued and the same speed was used for all the experiments.

The bisections may be made either with the sounds arranged in increasing order of intensity or in decreasing order; it was found that the first order gave more reliable results in that estimations were easier to make; this order therefore was used throughout the work. The commutator is therefore rotated in the direction shown in fig. 1, the repeated sequencies being sectors A, B, C, E, *i.e.*, oscillations from decibel box 1, from wheel resistance, from decibel box 2, silence, each portion occupying the same time, one-quarter of a revolution.

The intensities X_1 and X_5 corresponding to the first pair of sensations to be bisected are fixed at the convenient values of 20·3 decibels above the threshold on box 1, and 58·6 decibels above the threshold on box 2, and a set of bisection experiments made for each observer. The value of $R_{B'}$, fig. 1, is fixed at a value which will bring the wheel reading of the bisection to a convenient part of the scale, that is neither too high nor too low. A set of experiments consists of 10 consecutive observations made on each observer; this may take from 5 to 10 minutes. After a rest the observer continues, making 100 observations in all. The threshold is fixed on the resistance R_T before each set of 10 observations; when this is being done decibel box 1 is in action at the 0 decibel setting.

A complete set of 100 observations for the first observer is given in Table I, the numbers referring to wheel readings.

From the graph, fig. 5, 145·7 is 4·2 decibels below the 200° datum line. Also the standard deviation in decibels is 1·74. This is found from the slope of the curve at the point under consideration.

The meaning of 4·2 decibels below the datum line must next be found in terms of decibels above the threshold. To do this a series of "just perceptible difference" experiments is performed, to find what setting on the wheel resistance is equivalent to 44·9 decibels on box 1. This is chosen because it happens to be a setting on the decibel box which gives convenient readings. (The actual procedure of the "just perceptible difference" method for the determination of equal intensities is explained in the Appendix.)

The result of this standardization is that 44·9 decibels above the threshold is 3·0 decibels below the datum line of fig. 5, in other words our datum line, for the particular value of $R_{B'}$ chosen, is $44 \cdot 9 + 3 \cdot 0 = 47 \cdot 9$ decibels above the

threshold. Therefore the bisection value of 4·2 decibels below the datum line is 43·7 decibels above the threshold.

The result of the above is therefore that when 20·3 decibels and 58·6 decibels are bisected the value obtained is 43·7 decibels with a standard deviation of 1·74 decibels.

We have bisected X_1 and X_5 to find X_3, but a difficulty now arises when we try to bisect X_1 and X_3 to find X_2 and to bisect X_3 and X_5 to find X_4. The difficulty is that the value of X_3 obtained by the first bisection experiment will not in general be found on either of the decibel boxes. The way out is to take two values, one on either side of X_3 and calculate what the result should be assuming proportional parts. Thus we can determine X_2 and X_4 but

Table I—First observer, right ear, $R_{B'} = 1500$ ohms

Date	23.10.33	23.10.33	23.10.33	23.10.33	24.10.33	24.10.33	24.10.33	24.10.33	25.10.33	25.10.33
Time	11.30	12.15	14.15	16.00	10.00	11.30	14.45	15.15	10.00	11.15
	154·5	135·0	155·5	159·0	131·0	138·0	148·5	170·5	198·0	189·0
	153·5	124·5	145·0	141·0	115·5	132·0	164·0	130·0	141·0	136·0
	131·0	132·0	134·0	144·0	116·0	138·0	188·0	121·5	146·5	174·0
	132·5	154·0	108·0	160·0	132·0	110·5	181·0	111·0	159·5	152·0
	153·0	138·0	144·0	134·0	120·0	128·5	159·0	162·0	169·5	135·0
	162·0	155·5	139·0	133·5	136·0	130·0	172·5	135·5	154·0	171·0
	164·0	143·5	133·0	145·0	124·5	163·5	163·0	130·5	157·0	161·5
	177·0	144·0	145·5	150·0	121·0	101·5	156·0	144·0	180·5	154·0
	178·0	152·5	159·0	142·5	110·0	135·0	180·5	146·5	144·5	128·0
	171·0	117·5	134·5	141·5	132·0	122·0	167·0	127·0	175·0	150·0

The observations are taken to within 0·5 division.
Mean of 100 observations = 145·7. Standard deviation = 19·55.

when we come to bisect these and find X'_3 it is necessary to make four sets of observations combining two on either side of X_2 and X_4. The method of calculating the results will be explained later in the paper.

It was thought advisable to intersperse some sets of observations of the original bisection in between the other experiments in order to keep a control on the conditions.

These interspersed observations can be included when making the final computations.

A summary of all the results of the bisection experiments is given in Tables IIA and IIB. Each experiment represents the mean and standard deviation of 100 observations. The experiments in each table are given in the order in which they were performed.

Column 1 indicates the serial number of the experiment. Column 2 indicates the date. Column 3 indicates the decibels bisected measured above the threshold. Column 4 indicates $R_{B'}$, fig. 1, in ohms. Column 5 indicates

wheel reading of bisection, average of 100 observations. Column 6 indicates bisection in decibels above 200° datum line from fig. 5. Column 7 indicates reference point on box 1 in decibels above the threshold. Column 8 indicates 200° datum line in decibels above reference point given in column 7. Column 9 indicates 200° datum line in decibels above the threshold; column 7 + column 8. Column 10 indicates bisection in decibels above the threshold; column 6 + column 9. Column 11 indicates standard deviation of bisection in wheel readings. Column 12 indicates standard deviation of bisection in decibels.

Table IIA—Observer A, right ear

	2	3	4	5	6	7	8	9	10	11	12
1	2.10.33	20·3 and 58·6	2000	149·8	−3·8	48·7	0·8	49·5	45·7	30·77	2·76
2	5.10.33	20·3 ,, 40·0	200	211·9	+0·7	30·3	0·9	31·2	31·9	20·38	1·125
3	11.10.33	20·3 ,, 44·5	500	149·5	−3·8	35·4	4·45	39·85	36·0	15·44	1·295
4	16.10.33	40·5 ,, 58·6	4000	184·8	−1·0	48·7	5·5	54·2	53·2	17·72	1·215
5	18.10.33	44·9 ,, 58·6	4000	196·5	−0·3	48·7	5·5	54·2	53·9	11·66	0·735
6	23.10.33	20·3 ,, 58·6	1500	145·7	−4·2	44·9	3·0	47·9	43·7	19·55	1·74
7	25.10.33	30·3 ,, 49·3	1500	148·9	−3·8	44·9	3·0	47·9	44·1	10 55	0·885
8	27.10.33	35·4 ,, 49·3	1500	164 0	−2·6	44·9	3·0	47·9	45·3	7·85	0·60
9	31.10.33	20·3 ,, 58·6	1500	171·2	−2·0	44·9	3·0	47·9	45·9	20·52	1·035
10	3.11.33	30·3 ,, 53·4	1500	190·8	−0·55	44·9	3·0	47·9	47·4	13·89	0·93
11	6.11.33	35·4 ,, 53·4	1500	223·3	+1·35	44·9	3·0	47·9	49 2	10·83	0·545
12	9.11.33	20·3 ,, 58·6	1500	142·0	−4·35	44·9	3·0	47·9	43·6	16·81	1·52

Table IIB—Observer B, right ear

1	2	3	4	5	6	7	8	9	10	11	12
1	3.10.33	20·3 and 58·6	2000	95·0	−11·0	48·7	0·8	49·5	38·5	9·35	1·89
2	9.10.33	20·3 ,, 34·8	200	165·4	−2·5	30·3	0·9	31·2	28·7	13·71	1·055
3	13.10.33	20·3 ,, 40·0	500	112·7	−8·2	35·4	4·45	39·95	31·8	6·18	0·93
4	17.10.33	35·4 ,, 58·6	4000	148·5	−3·9	48·7	5·5	54·2	50·3	14·76	1·26
5	19.10.33	40·5 ,, 58·6	4000	175·4	−1·6	48·7	5·5	54·2	52·6	13·17	0·955
6	24.10.33	20·3 ,, 58·6	1500	107·5	−8·95	44·9	3·0	47·9	39·0	10·54	1·73
7	27.10.33	30·3 ,, 49·3	1500	144·9	−4·2	44·9	3·0	47·9	43·7	8·85	0·76
8	30.10.33	35·4 ,, 49·3	1500	155·0	−3·35	44·9	3·0	47·9	44·6	13·22	1·06
9	1.11.33	20·3 ,, 58·6	1500	115·5	−7·7	44·9	3 0	47·9	40·2	9·51	1·375
10	4.11.33	30·3 ,, 53·4	1500	175·9	−1·7	44·9	3·0	47·9	46·2	13·50	1·025
11	7.11.33	35·4 ,, 53·4	1500	201·2	+0·8	44·9	3·0	47·9	48·7	12·89	0·77
12	10.11.33	20·3 ,, 58·6	1500	102·6	−9·3	44·9	3·0	47·9	38·6	10·02	1·705

The results required for the final calculations are those in column 10, the "bisections" measured in decibels above the threshold, and those in column 12, the standard deviations of the bisections. For observer A the results are calculated thus. Experiments 1, 6, 9, and 12 for the bisection of 20·3 and 58·6 decibels above the threshold are first combined together, the mean being found and the standard deviation of the mean determined from the formula

$$n\sigma^2 = n_1(\sigma_1^2 + d_1^2) + n_2(\sigma_2^2 + d_2^2) + n_3(\sigma_3^2 + d_3^2) + \ldots, \qquad \text{(i)}$$

where

n is the total number of observations.

$n_1, n_2, n_3, \ldots$, the number of observations in each group.

σ the required standard deviation of the mean.

$\sigma_1, \sigma_2, \sigma_3, \ldots$, the standard deviation of each group.

$d_1, d_2, d_3, \ldots$, the differences between the mean and the mean of each group.

For observer A this gives a mean of 44·72 decibels with a standard deviation of 2·161 decibels.

We have now found X_3, the bisection of X_1 and X_5, and must next find X_2 the bisection of X_1 and X_3, and X_4, the bisection of X_3 and X_4. Two expressions will be required—

Let the bisection of D decibels and A decibels be a decibels,

" " D " C " c "

all measured above the threshold. Then we require b decibels, the bisection of D decibels and B decibels, B is not very different from either A and C. Assuming proportional parts since the differences between A and B and between B and C are small,

$$\frac{(b-a)}{(c-b)} = \frac{(B-A)}{(C-B)},$$

which gives

$$b = \frac{c\,(B-A) + a\,(C-B)}{(C-A)}. \tag{ii}$$

Let the standard deviation of a be α decibels.
Let the standard deviation of b be β decibels.
Let the standard deviation of c be γ decibels.
Let the standard deviation of B be π decibels.

A and C are fixed, not being experimental values, they will therefore have no deviations.

The general formula for the standard deviation of a derived function is :—

if $\quad G = f(x, y, z, \ldots)$

p_1, p_2, p_3, the standard deviations in $x, y, z, \ldots$

P the standard deviation in G;

then

$$P^2 = \left(\frac{\partial f}{\partial x}\right)^2 p_1^2 + \left(\frac{\partial f}{\partial y}\right)^2 p_2^2 + \left(\frac{\partial f}{\partial z}\right)^2 p_3^2 + \cdots,$$

therefore from expression (ii) we obtain

$$\frac{\partial f}{\partial a} = \frac{C - B}{C - A}, \qquad \frac{\partial f}{\partial c} = \frac{B - A}{C - A}, \qquad \frac{\partial f}{\partial B} \quad \frac{c - a}{C - A},$$

which gives

$$(C - A)^2 \beta^2 = (C - B)^2 \alpha^2 + (B - A)^2 \gamma^2 + (c - a)^2 \pi^2. \qquad \text{(iii)}$$

We can now find the bisection of 20·3 and 44·7 decibels for observer A.

From experiment 2, Table IIA, the bisection of 20·3 and 40·0 decibels is 31·9 decibels, standard deviation 1·125. From experiment 3, Table IIA, the bisection of 20·3 and 44·5 decibels is 36·0 decibels, standard deviation 1·295. The values to substitute in expressions (ii) and (iii) are :—

$$D = 20{\cdot}3, \quad A = 40{\cdot}0, \quad B = 44{\cdot}7, \quad C = 44{\cdot}5, \quad a = 31{\cdot}9,$$
$$c = 36{\cdot}0, \quad \alpha = 1{\cdot}125, \quad \pi = 2{\cdot}161, \quad \gamma = 1{\cdot}295.$$

which gives

$$b = 36{\cdot}2 \text{ from expression (ii)}$$

and

$$\beta = 2{\cdot}39 \text{ from expression (iii)},$$

this is the value of X_2 and its standard deviation.

In a similar way from experiments 4 and 5 in Table IIA

$$X_4 = 53{\cdot}8 \text{ with a standard deviation } = 0{\cdot}78.$$

In order to find X'_3 and its standard deviation it is necessary to make three applications of the formulæ (ii) and (iii) above. From experiments 7 and 8 combined together we obtain bisection of 49·3 and 36·2 decibels giving 45·5 decibels with a standard deviation of 0·90 ; from experiments 10 and 11 the bisection of 53·4 and 36·2 decibels gives 49·5 decibels with the standard deviation 1·06. Combining these we obtain the result that the bisection of 53·8 (X_4) and 36·2 (X_2) gives for X'_3 the value 49·9 with a standard deviation 1·40.

The results for observer A can be summarized thus :—

$X_1 = 20{\cdot}3$, $\sigma = 0{\cdot}0$		
	$X_2 = 36{\cdot}2$, $\sigma = 2{\cdot}39$	
$X_3 = 44{\cdot}7$, $\sigma = 2{\cdot}16$		$X'_3 = 49{\cdot}9$, $\sigma = 1{\cdot}40$
	$X_4 = 53{\cdot}8$, $\sigma = 0{\cdot}78$	
$X_5 = 58{\cdot}6$, $\sigma = 0{\cdot}0$		

The results for observer B obtainable in a similar way from Table IIB can be summarized :—

$X_1 = 20 \cdot 3$
$\sigma = 0 \cdot 0$

$X_2 = 31 \cdot 1$
$\sigma = 1 \cdot 28$

$X_3 = 39 \cdot 1$
$\sigma = 1 \cdot 82$

$X'_3 = 45 \cdot 6$
$\sigma = 1 \cdot 07$

$X_4 = 51 \cdot 95$
$\sigma = 1 \cdot 13$

$X_5 = 58 \cdot 6$
$\sigma = 0 \cdot 0$

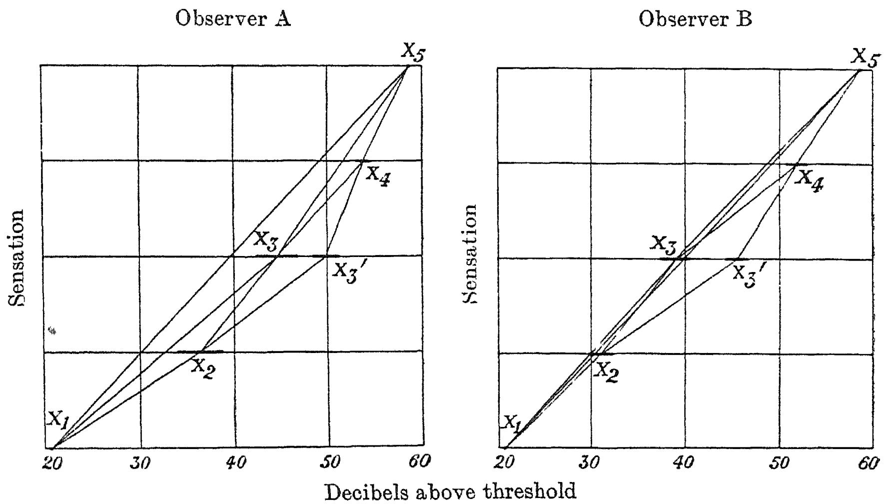


FIG. 6

The results are shown diagrammatically in fig. 6. The stimulus in decibels above the threshold, is here plotted against what may be called the sensation. The points X_1 and X_5 are taken as two fixed levels on the sensation scale we are attempting to construct, and X_3 is the bisection of these two sensations. It is therefore placed half-way between them in the vertical direction, the same plan is adopted in placing X_2 and X_4. When we come to X'_3, this must be placed on the half-way line between X_2 and X_4, that is on the same level as X'_3. The lengths of the thick horizontal lines indicates twice the standard deviations of the respective points. An examination of the standard deviations of X_3 and X'_3 indicate that these two points must be considered to differ significally from each other. Each of the diagrams is a summary of 1200 observations.

Conclusion

These experiments were performed to test the possibility of building a scale of sensation in the case of sound. The observations were intentionally made on two observers and statistical methods applied to the results of each observer separately. The observations were also confined to pure notes of the same frequency, the conditions being thus reduced to the simplest possible. The results indicate that it is not possible to build a scale of sensation even under these very limited conditions.

Various attempts have been made to build a scale of sensation by such methods as estimating when one sound is half as loud as another. In some preliminary work we found this very difficult to judge, in fact, it was very difficult to know exactly what one was doing. As for adjusting one sound to be one-quarter or any other fraction of another, this seemed quite impossible. It may be possible to average observations from a large number of observers and obtain average measurements, but this seems a questionable procedure. It is surely better to consider each individual ear separately. No one would think of drawing conclusions as to the response of telephone ear pieces by taking a large number of instruments of different shapes and sizes and sensitivities and averaging all the measurements made on them. This is not to say that such methods are of no value, but for the purpose in hand, that of finding if sensation responses can be measured, they are of little use.

A corollary of the general proposition that a scale of loudness cannot be constructed is that there is no " decibel " scale of sensation. This is an important particular case which merits special attention. The decibel is a convenient way of expressing physical intensities of sound, but great caution must be taken in its use in physiological work, since it is very easy to assume a scale of sensation without realizing that one is doing so.

These remarks must not be taken to mean that quantitative estimation of loudness is impossible. In fact our results illustrate one method of making such estimates. But this is a different matter from placing sensations on a definite scale as functions of stimuli; in general, a self-consistent series cannot be combined with a second equally self-consistent series to form a consistent whole. That a sensation scale cannot be realized may not be due to anything intrinsically wrong in the conception of such a scale, so that it may be worth while to look for some explanation of the failure. One factor must be borne in mind in any such attempt: the phenomenon of adaptation, but adaptation in the case of hearing is a very elusive phenomenon. Most of the work which

has been done in the case of hearing has been concerned with fatigue rather than adaptation. But whether the conception of a scale of sensation is legitimate or not, any further work done on auditoy.sensation must start with the reservation that even in the simplest possible circumstances loudness is not an unvarying function of stimulus.

In conclusion the author wishes to thank the Medical Research Council for grants which have made this work possible, and also to express his appreciation for the help given by Dr. J. H. Shaxby, in whose laboratory the experiments were performed.

Summary

The paper consists of an investigation of the possibility of building a scale of sensation for intensity of sounds. The work is confined to pure tones and the psychophysical judgment made is to adjust the intensity of a sound to make its loudness appear to lie equally between the loudnesses of two other notes. The three notes are all of the same frequency and are presented successively to the ear in order of increasing loudness. This is called the method of " bisection." The apparatus is electrical, consisting of a pure tone heterodyne oscillator followed by an amplifier and telephone which is the source of sound. Means are provided for intensity control in decibels above the threshold and a commutator is used to give the three successive notes. The purity of the note obtained is examined by a cathode ray oscillograph fitted with a linear time sweep circuit. The calibration of the apparatus is described in the Appendix.

It is shown that if loudnesses corresponding to the intensities X_1 and X_5 are bisected to give X_3 and the two ranges obtained further bisected to give X_2 and X_4 respectively, then on bisecting X_2 and X_4 to give X'_3, this last intensity is not identical with X_3 and the discrepancy lies outside the experimental error.

The conclusion is that a scale of sensation cannot be built up, and in particular the decibel is not a measure of auditory sensation but simply a method of specifying intensity, that is physical stimulus.

Appendix

Calibration of the Decibel Boxes

The decibel boxes were constructed from wire-wound resistances, the required values being calculated to give steps of 5 decibels, ranging from 0 to 70 decibels. These resistances have a certain amount of inductance and capacity and therefore will not give accurate values for frequencies such as 800 cycles per second; the calculated decibels will only be correct at zero frequency. Of course methods of winding are well known which reduce self-inductance and self-capacity to an amount which can be neglected provided the frequency is not too high, but for the highest frequencies calibration will always be necessary.

The method used for the calibration of the decibel boxes is to compare them with the wheel resistance R_B, fig. 1. This is calibrated as explained in the text, but it may be noted here that the method adopted for the calibration of this resistance may be carried out with alternating current of a frequency of 800 if desired, since an alternating current measuring instrument, the electrostatic voltmeter, is used as the indicating instrument. To return to the decibel boxes, the calibration is performed acoustically at the required frequency of 800; the apparatus shown in fig. 1 can be used without modification.

The commutator sequence, assuming box 1 to be in action and box 2 out of action, that is, set at $-\infty$ decibels, is therefore for the direction of rotation shown, sectors A, B, C, E, *i.e.*, oscillations from decibels box 1, from wheel resistance, silence from decibel box 2 and from earth connection; the silence period thus occupying half of the total time of a period of rotation. The speed of rotation of the commutator is of no prime importance, it was therefore left at the same value as that used in the main research.

Now $R_{B'}$ is fixed at a suitable value to make the graph of fig. 5 cover two steps of 5 decibels, for example the 40, 45 and 50 nominal decibel ranges on box 1. The sound obtained from the wheel resistance is made as nearly as possible equal in loudness to that obtained from the 40 decibel (nominal) setting of box 1 by the observer in the sound proof room pulling the string attached to the wheel resistance, and the reading of the wheel taken by the outside assistant in the usual way. The process is repeated for the 45 and 50 decibel settings of box 1. On referring to fig. 5 we can read off the actual

differences in decibels between these settings and obtain a calibration covering the 40 to 50 decibel range. The process can then be repeated by altering $R_{B'}$, fig. 1, and the 45, 50 and 55 decibel range covered. By starting at the bottom end of the box, *i.e.*, at 0 decibels and repeating the process it is possible to calibrate the box up to 65 decibels; the presence of R_D prevents the top end, 70 decibels, being reached. This does not matter since the most intense sound used in this work is 60 decibels above the threshold.

It may be noted that in these calibration experiments we are at liberty to choose any intensity of the sound given by the telephone, therefore R_T and R_0 were adjusted from time to time to give a convenient loudness. The best intensities to use will be evident from the next paragraph.

A slight elaboration of the process was found to give much greater accuracy. It is well known that there is a psycho-physiological limit to the accuracy with which two sounds can be adjusted to equality by a subjective method; if they are nearer together than the differential limen they will sound equally intense. The following "just perceptible difference" method was therefore adopted. With the commutator rotating in the direction shown the wheel resistance is adjusted to make the sound from it appear just appreciably louder than that from decibel box 1. The direction of rotation of the commutator is then reversed and the wheel resistance adjusted to make the sound from the decibel box appear just perceptibly louder than the sound from the wheel resistance. It will be noted that the second sound is always louder than the first. The mean of the two settings of the wheel resistance is taken to represent the point of equality. The value thus found will be independent of the differential threshold of any particular observer; it is well, however, to work in a region where the "just perceptible difference" is small, *i.e.*, to avoid using very quiet or very loud notes. This can be done by adjusting R_T and R_0.

The threshold in the actual "bisection" experiments was always fixed at 0 decibels on box 1. The calibration of both decibel boxes was therefore made from this point as zero. The final results of the calibrations are shown in Table III correct to 0·1 decibels. This is as accurate as the figures justify and is sufficiently precise for any experiments in physiological acoustics.

These calibrated settings on the decibel boxes can now be used as reference points; readings on the wheel resistance, whatever the value of $R_{B'}$ can always be referred to any suitable point on decibel box 1 by a short series of "just perceptible difference" experiments as described above

The decibels are measured above the 0·0 setting in box 1.

Table III—Calibration of Decibel Boxes

Box setting, nominal	Decibel box 1	Decibel box 2
0	0·0	−0·3
5	5·0	4·7
10	10·3	9·7
15	15·3	15·1
20	20·3	20·1
25	25·4	25·0
30	30·3	29·8
35	35·4	34·8
40	40·5	40·0
45	44·9	44·5
50	48·7	49·3
55	53·4	53·4
60	58·1	58·6
65	62·8	63·3

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An Experimental Investigation of the Measurability of Visual Sensation

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(Communicated by T. Graham Brown, F.R.S.—Received May 12, 1934)

The following paper deals with the question of visual intensity (brightness) along lines similar to those in the previous paper upon auditory sensations.

It may be noted at once that whenever the physicist has to depend on the direct quantitative estimates of sensation instead of the indications of some instrument such as a galvanometer, he tries to avoid the problem by reducing the measurement to a judgment that two sensations are equal. Probably the best known example of this is photometry. The accuracy of the "measurement" found in this way depends ultimately on a sensory judgment, but the actual determination does not.

It is not the purpose of this paper to enter into theoretical discussion; the work was undertaken from the following standpoint. The assumption is made that there is nothing intrinsically wrong with the use of psychophysical estimations in attempting the measurement of sensation. One of the standard criticisms of the Weber-Fechner law is that the "just-perceptible-differences" are not of necessity equal increments of sensation. Similar criticisms have been made concerning other methods of estimation (Campbell and others, 1933), and they can equally well be applied to the present work. It will be assumed, then, that it is worth while making experimental observations to see if it is possible to measure "sensation" without considering the more theoretical aspects of the problem. If it can be shown that it is not possible to make a sensation scale by direct experimentation, then the case against the measurability of sensation is made certain.

In the previous paper, an experimental research was made into the measurability of auditory sensation. The result of this work was to show that a scale of sensation for sound could not be constructed under the simplest possible conditions.

Since most of the discussion on scales of sensation has been concerned with visual stimuli (Richardson, 1929; Maxwell, 1929; Smith, 1930; Richardson

and Maxwell, 1930; Richardson, 1932; Guild, 1932; Campbell and others, 1933), the work on hearing was followed up by making analogous experiments in the case of vision. In hearing, the work was done on the "loudness" of sounds and the intensities of the causal stimuli. When we come to vision, however, we have a wider choice; it is possible to work with "hue," "saturation," or "brightness." Most of the work has been done on the estimation of colours such as the amount of redness and whiteness in a given pink, but it was thought advisable in the first place to use white light and confine the attention to brightness which can be correlated unequivocally with the physical intensity.

In hearing we are compelled to present two or more stimuli consecutively, if they are of the same frequency, but in the case of vision we can either present the stimuli consecutively on the same part of the retina, or present them simultaneously on different parts of the retina. The first method would be analogous to the case of hearing, but it is more difficult to realize experimentally than the second, also the latter is more usual and is therefore the one adopted in this work.

The psychophysical judgment made was that used in the previous paper on hearing and there discussed in detail. This is called the method of "bisection" and will be very briefly described here. Suppose three visual stimuli, A, B, and C, to be presented side by side, the stimuli differing only in one way, that of intensity, A being more intense than B, and B more intense than C. Then if means are provided for varying the brightness of B, it is possible to adjust B so that it appears equidistant in respect of brightness from A and C. As with hearing a large number of observers were tested to see if they could make this estimate, and in every case they were satisfied that it could be done. The two intervals A to B and B to C thus obtained are again bisected to find two further brightnesses which may be designated the "¼-way" and "¾-way" sensations. Finally the interval between these "¼-way" and "¾-way" sensations is bisected and this final bisection should give the original "½-way" sensation if there is to be a possibility of correlating "sensations" with measurements of the intensities of stimuli. If there is no such agreement, and if the discrepancy is significant, being outside the overlapping of the probable errors of the experiment, then there cannot be a scale of brightness founded on such intervals of brightness.

Three circular discs of light were used as stimuli, and their size and relative positions depend to a large extent on experimental expediency. The apparatus to be described was the final form of several attempts to realize a convenient

arrangement. Three points must be borne in mind: the intensities of the three discs of light must be easily controlled and measured, the discs must be brought reasonably near together, and ample ranges of intensities must be available.

In order to specify in a convenient way the large changes of intensity, resource is made to a logarithmic scale, just as the decibel scale is used for the same reason in the case of hearing. The " density " of a light filter is defined by the relation

$$d = \log_{10} \frac{1}{k},$$

where d is the density and k is the fraction of the incident light transmitted by the filter.

The filter may consist of a glass flat with a film which absorbs all wavelengths of the visible spectrum equally, such as the Wratten neutral filters, or alternatively it may consist of a rotating sector, the angular aperture of which gives the corresponding density from the above formula. It is most convenient to construct such rotating sectors with angles not measured in degrees but in percentages of total transmission. Thus a sector consisting of two right angles transmission would be called a 50 sector, and the corresponding density would be $\log_{10} 100/50 = 0 \cdot 301$. The densities for any sector percentage can be found directly from a table of logarithms of reciprocals.

In practice, it is often convenient to combine in one apparatus both rotating sectors and neutral glass filters. This procedure is adopted in the present work. The rotating sectors are more straightforward in use than the glass filters because the determination of density is simply a matter of geometry, but they cannot be made of sufficient accuracy for large densities. For instance, for a density of $2 \cdot 00$, since it is best to make the sectors with two " blades," each aperture will have an angle of 1/100 of two right angles, which is too small to cut with sufficient accuracy.

The glass filters used were standardized in terms of the rotating sectors. One important point was always observed: filters and sectors to produce a given density were always chosen in the same way. Suppose, for example, we require a density of $1 \cdot 000$ which is equal to a transmission of 10%. This can be arranged either by a $d = 1 \cdot 000$ filter and no sector, or by a $0 \cdot 301$ filter and a $0 \cdot 699$ sector, *i.e.*, one with an angular transmission of 20%. The second method, being the most suitable for the first experiment in which this density was required, was therefore used throughout the research. The final con-

clusions of the work do not depend on the accuracy of the calibration of the neutral filters, although for the sake of completeness this was done as carefully as possible.

It will be noticed that the analogy between the density of the light filters for the specification of light intensities and the decibel system for the definition of sound intensities is not complete since they go in opposite directions. The more intense the sound the greater its measure in decibels, but the more intense the light the smaller is its density number. In both systems the difficulty of making absolute measures in physical units may be avoided; in the case of the decibel it is usual to take the threshold of hearing as the zero, but in the present work on vision an arbitrary intensity was taken as the reference level.

Description of the Apparatus

Three circular discs of light are to be presented to the eye and the intensity of the light from each disc must be under control by a system of neutral filters and rotating sectors; the middle disc (that of intermediate brightness) must be controlled by the observer while the sectors are revolving. Also the discs of light are to be brought as near together as possible to aid in the judgment to be made. The nearest distance between the discs which was found to be practicable was such that the distance between centres was equal to twice the diameter of the discs.

The intensity control is shown in fig. 1. The board LLLL has four apertures A, B, C, and D through which the light beams pass. (Only three of these, namely A, B, and C, are used in the present work but the apparatus was constructed with four for another purpose.) Behind each aperture can be placed a neutral filter and in front is arranged the system of pulleys shown which drive the rotating sectors. Two sectors are shown, sector U having a fixed aperture and sector V a varying aperture. This latter type of sector can be mounted on the rocking arms R_1 and R_2 and the effective aperture can be altered while it is rotating. The system of pulleys is designed to enable the rocking arms to be moved up or down by pulling on the strings shown dotted in the diagram. The slots S_1 and S_2 are made in the board LLLL to take pointers projecting behind the board and attached to R_1 and R_2. These pointers work over scales attached to the back of the board. During the experiments only R_2 was required to be moved and it was usual to fix R_1 and place on it a sector of fixed aperture.

The shape of the variable aperture sectors similar to V is such that the angle of the aperture bears a linear relationship to the radius and therefore a calculable density range is covered by a sector of this type when it is moved across the beam passing through B. A set of sectors was constructed giving apertures of 0-30, 10-40, 20-50, etc., per cent. transmission and the position read upon the scale at the back of the board gives the actual percentage transmitted. The scale reads from 0 to 30 from above downwards, and to its reading must be added the minimum percentage transmitted by the sector. The system of rotating sectors is driven by the pulley P which is connected to a motor not shown in the figure. The speed of the sectors must be made sufficiently great to avoid "flicker."

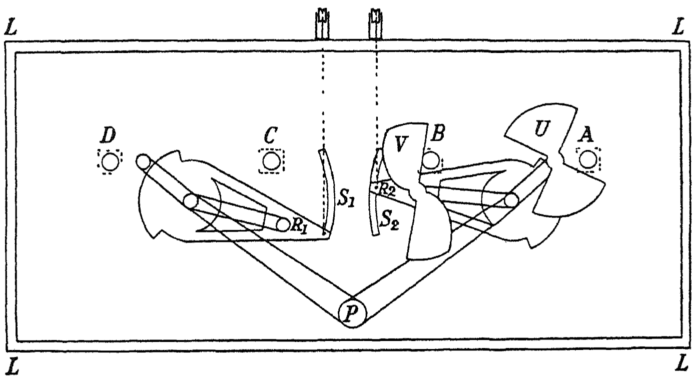


FIG. 1

The board LLLL is set up vertically; a plan of the complete apparatus is shown in fig. 2. (Here only the three apertures used are shown.) Behind the board are placed the lamp boxes M_A, M_B, and M_C. A diagram of one of these is shown in fig. 2*b*. The lamp T is a 60 watt gas-filled daylight lamp supplied from the direct current mains. When the apparatus was first set up "flickering" appeared which was traced to stroboscopic effects due to the use of alternating current; this was completely eliminated when a change was made to direct current. The light from T is diffusely reflected from the screen N which can be rotated around a horizontal axis. The amount of diffused light can be controlled to some extent by the angle made to the horizontal by the screen N. A pointer attached to N works over a scale outside the lamp box and is used to adjust the respective light beams to be equal in intensity. The three lamp boxes are made as similar as possible and the lamps are selected to be very nearly the same from a batch of supposed equality.

After passing through the filters and sectors the two outer beams are reflected from the system of mirrors RRRR into the light-proof box FFFF. Each of these four mirrors is provided with a universal adjustment to make possible the final alignment of the apparatus. The light from the central aperture B passes straight through the centre of the apparatus without reflection. In order to avoid colour differences between this central beam and the two outer beams due to the double reflections from the mirrors, a double reflection of the central beam is arranged before it reaches the board LLLL. This is done by lifting the central lamp box M_B above the level of the outer lamp boxes M_A and M_C and placing two mirrors Q, as in a periscope and shown in plan in fig. 2, to bring the beam to the aperture B. The total lengths of the three light beams are made equal.

The three discs of light seen by the eye are formed by the three apertures *a*, *b*, and *c* cut in a brass plate fitted to the end of FFFF; it will be seen that the final definition of these discs is made after all the reflections have taken place, and therefore no trouble is experienced from the front surface reflections of the mirrors. The interior of FFFF is painted black.

The eye is placed at E and views the three discs of light through the convex lens X and neutral wedge W. The result is that each disc appears at a distance of 82·3 cm from the eye and subtends an angle of 38′. The neutral wedge W, which can be moved at right angles to the plane of fig. 2, is used to control the intensities of all the beams together.

Calibration and Adjustment of the Apparatus

Two preliminary sets of experiments must be made before commencing the actual "bisections":—

1. The three beams must be made of equal intensity.
2. The neutral filters must be standardized.

Equality of intensity is obtained by the use of the scales and pointers attached to the screens N, fig. 2*b*. Photometric comparisons between the beams are made using the rotating sectors and calibration curves showing the variation of light from each screen N with its position are made. Positions can then be selected which will give equality. These are checked from time to time during the course of the work.

The standardization of the filters is made in terms of the rotating sectors. Supposing the beams from A and B adjusted for equality, the 0·301 nominal filter is placed in the beam A and the 30-60 sector in the beam B and a series

of observations taken to make the two beams appear equal in brightness. The position of the 30-60 sector is adjusted by pulling the string attached to R_2, fig. 1. The average of a series of observations may be taken to give the percentage cut off by the sector, and the corresponding " density " found. This must be equal to the " density " of the filter. By combining the filter thus standardized with a sector on the aperture B it is possible to proceed with the standardization of filters of higher densities, the latter being placed in the beam A. In this way the use of small angle sectors is avoided.

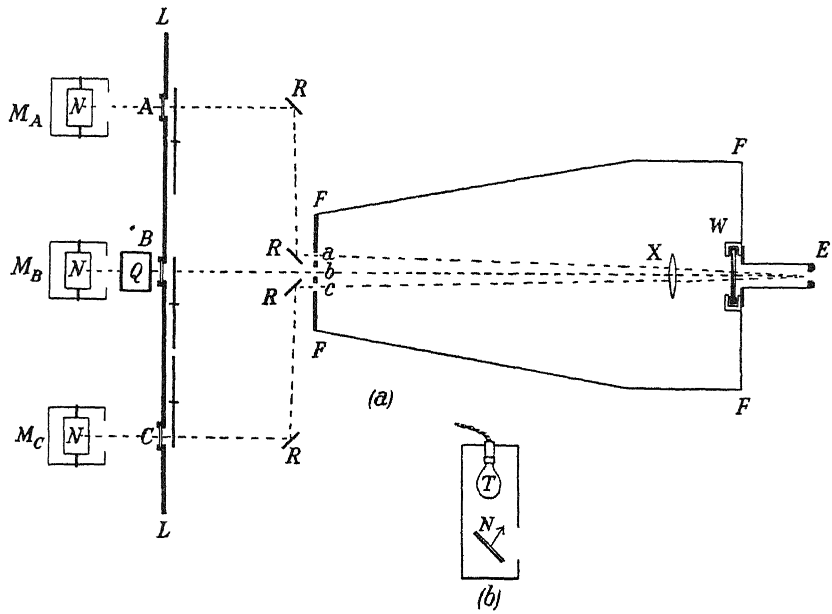


FIG. 2

The white light standardization of the three filters required in the present work resulted as follows :—

Filter density (nominal)	Filter density (actual)
0·301	0·321
1·000	1·059
2·000	2·026

The Experimental Method

Since the object of this paper is to test the possibility of building up a scale of sensation, it was thought advisable to make the conditions as favourable as possible ; if the results give a positive answer then the work may be extended to wider conditions, but if they give a negative answer the conclusion is all the more valuable since it is obtained under conditions which may be reason-

ably expected to be the most favourable. Medium intensities were therefore chosen for the three discs of light. Glare and also intensities which are so low as to approach the threshold of visibility are avoided. The neutral wedge W, fig. 2, was therefore set to transmit 1/100 of the total light incident upon it. The greater available intensities are useful if it is desired to use coloured filters. During the "bisection" experiments the laboratory was illuminated by subdued artificial light and the conditions were standardized as far as possible.

Suppose now that we desire to "bisect" the density 0·000 corresponding to no filter and no sector, and 2·026 corresponding to the 2·026 filter and no sector. We can either place the bright disc to the right or to the left. Both arrangements were used and the results worked out separately. With regard to the middle disc, it is found that a "bisection" can be made with a 0·321 filter and the 10-40 sector. In a particular case the average of 50 observations made in five sets of 10 gave a reading on the scale of 14·11%. Adding this to 10, the lowest transmission of the 10-40 sector, we find the percentage of light transmitted to be 24·11%. This corresponds to a density of 0·618. The density of the bisection is thus $0·321 + 0·618 = 0·939$. The procedure from this point is entirely analogous to that described in the previous paper on hearing, p. 103. The complete series of observations is given in the following tables, each experiment representing the average of 50 observations. In these

Table I—Observer A. Left eye. Brightest disc to the right. Density A and density C bisected to give density B

Experiment No.	Date	Density A	Density C	Density B	Standard deviation of B
1	12.1.34	0	2·026	0·912	0·0180
2	17.1.34	0	1·020	0·548	0·0290
3	19.1.34	0	0·844	0·412	0·0251
4	22.1.34	0	2·026	0·922	0·0456
5	25.1.34	1·020	2·026	1·371	0·0190
6	29.1.34	0·844	2·026	1·291	0·0186
7	31.1.34	0	2·026	0·937	0·0404
8	1.2.34	0·523	1·360	0·877	0·0399
9	2.2.34	0·523	1·281	0·795	0·0405
10	3.2.34	0	2·026	0·930	0·0553
11	5.2.34	0·398	1·360	0·747	0·0315
12	12.2.34	0·398	1·281	0·717	0·0282
13	13.2.34	0	2·026	0·862	0·0466

Table II—Oberver A. Left eye. Brightest disc to the left. Density A and density C bisected to give density B

Experiment No.	Date	Density A	Density C	Density B	Standard deviation of B
14	16.1.34	0	2·026	0·881	0·0407
15	18.1.34	0	1·020	0·471	0·0158
16	19.1.34	0	0·844	0·369	0·0186
17	23.1.34	0	2·026	0·825	0·0261
18	26.1.34	1·020	2·026	1·351	0·0172
19	29.1.34	0·844	2·026	1·250	0·0210
20	31.1.34	0	2·026	0·880	0·0509
21	2.2.34	0·523	1·360	0·814	0·0217
22	3.2.34	0·523	1·281	0·752	0·0243
23	5.2.34	0	2·026	0·827	0·0250
24	9.2.34	0·398	1·360	0·730	0·0189
25	12.2.34	0·398	1·281	0·626	0·0324
26	13.2.34	0	2·026	0·791	0·0215

Table III—Observer B. Left eye. Brightest disc to the right. Density A and density C bisected to give density B

Experiment No.	Date	Density A	Density C	Density B	Standard deviation of B
27	12.1.34	0	2·026	0·939	0·0436
28	17.1.34	0	1·020	0·634	0·0411
29	19.1.34	0	0·844	0·469	0·0288
30	22.1.34	0	2·026	0·895	0·0407
31	25.1.34	1·020	2·026	1·401	0 0268
32	29.1.34	0·844	2·026	1·310	0·0259
33	31.1.34	0	2·026	0·931	0·0489
34	1.2.34	0·523	1·360	0·939	0·0364
35	2.2.34	0·523	1·281	0·834	0·0253
36	3.2.34	0	2·026	0·947	0·0355
37	5.2.34	0·398	1·360	0·827	0·0291
38	12.2.34	0·398	1·281	0·750	0·0260
39	13.2.34	0	2·026	0·939	0·0700

Table IV—Observer B. Left eye. Brightest disc to the left. Density A and density C bisected to give density B

Experiment No.	Date	Density A	Density C	Density B	Standard deviation of B
40	16.1.34	0	2·026	0·931	0·0458
41	18.1.34	0	1·020	0·564	0·0417
42	22.1.34	0	0·844	0·431	0·0330
43	23.1.34	0	2·026	0·890	0·0337
44	26.1.34	1·020	2·026	1·367	0·0194
45	29.1.34	0·844	2·026	1·254	0·0186
46	31.1.34	0	2·026	0·908	0·0458
47	2.2.34	0·523	1·360	0·907	0·0331
48	3.2.34	0·523	1·281	0·804	0·0242
49	5.2.34	0	2·026	0·932	0·0386
50	9.2.34	0·398	1·360	0·762	0·0344
51	12.2.34	0·398	1·281	0·657	0·0268
52	13.2.34	0	2·026	0·904	0·0394

tables the density A and the density C are " bisected " to give the density B. The standard deviation of B is given in the last column.

The method of calculating the results will be briefly explained for Table I. Let the intensities originally bisected be called X_1 and X_5 and the " bisection " of the range X_1 to X_5 be X_3. Let the bisection of X_1 and X_3 be X_2 and the bisection of X_3 and X_5 be X_4. Finally let the bisection of X_2 and X_4 be X'_3. Then

$$X_1 = 0$$

and

$$X_5 = 2 \cdot 026.$$

It will be seen that experiments 1, 4, 7, 10, and 13 are repeated determinations of X_3. These are therefore combined together giving for X_3 the value

$$X_3 = 0 \cdot 918$$

with a standard deviation

$$\sigma = 0 \cdot 0565.$$

X_2 can be found from experiments 2 and 3, by assuming proportional parts for the small range of intensities concerned, giving

$$X_2 = 0 \cdot 470$$
$$\sigma = 0 \cdot 0476.$$

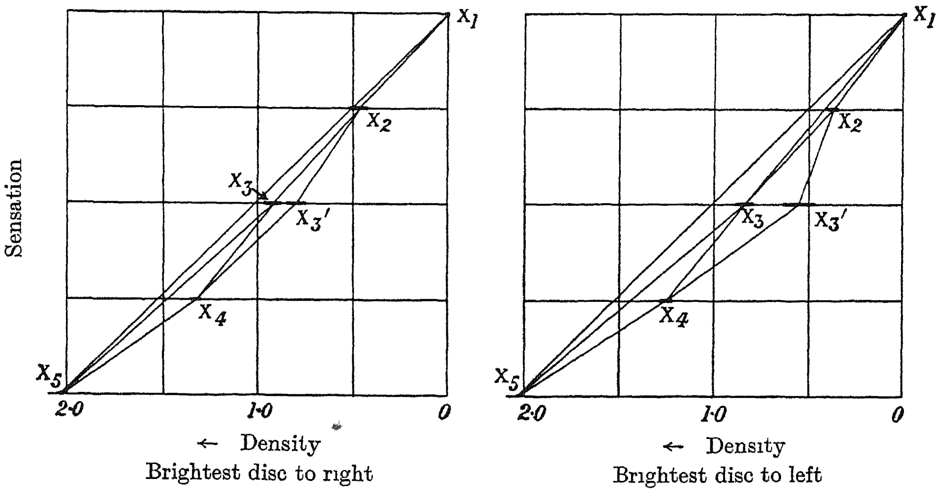
X_4 can likewise be found from experiments 5 and 6,

$$X_4 = 1 \cdot 326$$
$$\sigma = 0 \cdot 0290,$$

and finally by combining experiments 8, 9, 11, and 12, X'_3 is found to be given by

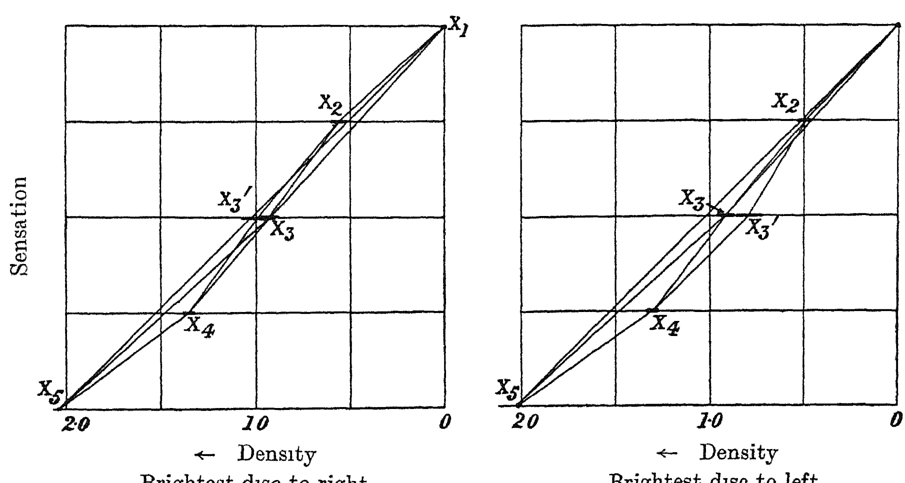
$$X'_3 = 0\cdot797$$
$$\sigma = 0\cdot0480.$$

The calculation is entirely similar to that explained in detail in the previous paper on hearing. The final results are given below and shown graphically in figs. 3 and 4.



Observer A left eye

FIG. 3



Observer B left eye

FIG. 4

1—Observer A. Left eye, brightest disc to right.

$X_1 = 0$
$\sigma = 0$

$X_2 = 0 \cdot 470$
$\sigma = 0 \cdot 0476$

$X_3 = 0 \cdot 918$
$\sigma = 0 \cdot 0565$

$X'_3 = 0 \cdot 797$
$\sigma = 0 \cdot 0480$

$X_4 = 1 \cdot 326$
$\sigma = 0 \cdot 0290$

$X_5 = 2 \cdot 026$
$\sigma = 0$

2—Observer A. Left eye, brightest disc to left.

$X_1 = 0$
$\sigma = 0$

$X_2 = 0 \cdot 366$
$\sigma = 0 \cdot 0340$

$X_3 = 0 \cdot 840$
$\sigma = 0 \cdot 0481$

$X'_3 = 0 \cdot 549$
$\sigma = 0 \cdot 0915$

$X_4 = 1 \cdot 247$
$\sigma = 0 \cdot 0352$

$X_5 = 2 \cdot 026$
$\sigma = 0$

3—Observer B. Left eye, brightest disc to right.

$X_1 = 0$
$\sigma = 0$

$X_2 = 0 \cdot 550$
$\sigma = 0 \cdot 0552$

$X_3 = 0 \cdot 930$
$\sigma = 0 \cdot 0525$

$X'_3 = 0 \cdot 984$
$\sigma = 0 \cdot 0842$

$X_4 = 1 \cdot 357$
$\sigma = 0 \cdot 0329$

$X_5 = 2 \cdot 026$
$\sigma = 0$

4—Observer B. Left eye, brightest disc to left.

$X_1 = 0$
$\sigma = 0$

$X_2 = 0 \cdot 484$
$\sigma = 0 \cdot 0421$

$X_3 = 0 \cdot 913$
$\sigma = 0 \cdot 0440$

$X'_3 = 0 \cdot 782$
$\sigma = 0 \cdot 0714$

$X_4 = 1 \cdot 300$
$\sigma = 0 \cdot 0314$

$X_5 = 2 \cdot 026$
$\sigma = 0$

Discussion of Results

The observations have intentionally been made on a small number of observers and also under very limited conditions. This may suggest that the conclusions to be drawn would only be of limited application. But it is only by taking very definite cases that any progress can be made, and the limited conditions used are just those for which, if ever, such judgments might be expected to be valid. Furthermore it is better to examine a few examples accurately than to spread the work over a large number of observers and average the results. To take such averages is to lose sight of the whole problem, the investigation of an eye regarded as an individual instrument.

Figs. 3 and 4 express the results in the following way. The densities are plotted horizontally, the greatest density, *i.e.*, the lowest intensity, being to the left, and vertically an attempt is made to plot the " sensation." X_1 and X_5 are bisected to give X_3 and this is shown as a sensation half-way between them. Similarly X_2 is shown half-way between X_1 and X_3, and X_4 is shown half-way between X_3 and X_5. The final bisection of X_2 and X_4, namely, X'_3, must therefore be shown at the same height as X_3. The criterion as to whether we can proceed by this method to build a scale of sensation will depend on whether X_3 and X'_3 can be considered to be one and the same point. The lengths of the thick horizontal lines are equal to twice the standard deviations of the respective points.

Considering fig. 3, it will be seen that in each case we must consider X_3 and X'_3 to be separate points, and therefore it can only be concluded that this method does not lead to a consistent way of placing sensations on a scale. The same conclusion must be drawn from fig. 4 when the brightest disc is to the left. When, however, the brightest disc is to the right, it does appear that

X_3 and X'_3 are sufficiently near together to make a scale of sensation possible. The density corresponding to X_2 being unusually large brings X'_3 much more to the left than in the other cases considered. We can only conclude that in this particular case, with the particular disposition of the apparatus, and over the ranges of intensity considered, a scale of sensation might be constructed. There can be no doubt, however, that this is an accidental case, and that if we only vary the conditions to include larger ranges of intensity, the scale would break down. As a general conclusion, the process used to place sensations on a scale is not successful and therefore sensations do not behave like a physical entity such as temperature. For if a similar experiment were performed in thermometry there can be no doubt that X_3 and X'_3 would coincide within very narrow limits.

Analogous results to those obtained in this paper were found in the previous paper on hearing. There again sensation could not be measured. It may be argued that no one would expect that sensation could be measured in a similar way to physical entities and this view has been held by many investigators in this field. The present work was done with the assumption that sensation may be set out on a scale of magnitude as functions of stimulus. It is found that as a general proposition this is not so.

Claims have been made in the case of hearing (Ham, 1932; Richardson and Ross, 1927) that it is possible to make a judgment when one sensation is half as loud or one-quarter as loud as another sensation. The writer of this paper found that this is a difficult judgment to make, perhaps because of his previous knowledge of the "decibel," but the same objection does not apply to the fact that he felt equal difficulty in the present work on vision. It should be pointed out that in the method of bisection used in the present paper and in the previous one on hearing the judgments seem to be quite independent of any preconceptions in the matter.

Some experiments done with the apparatus of the present paper threw an interesting light on the question of estimating when one sensation is half of another. Suppose our three sensations A, B, and C are presented in the usual way and the bisection made to make the intervals A to B and B to C equal. Then on decreasing the sensation C (the least bright) and keeping A fixed, the middle sensation B must also be decreased. What will happen when C approaches the threshold? The experiments showed that B would also approach the threshold. The shape of the curve obtained suggested that in the limit when any sensation above the threshold and any sensation below the threshold, that is no sensation, are bisected the result is a sensation just

about the threshold. In other words, half-way between any sensation and no sensation at all is the threshold of sensation. Whether this process is the same as judging when one sensation is half another it is not intended to say, but it does suggest that there may be some fundamental objection to the subjective estimation of sensations.

In a recent paper (Churcher, King and Davies, 1934) a scale of sensation for hearing has been built up by the process of estimating when one sound is half as loud as another. The spread of the results is greater than that in the present author's work on vision and hearing, and. of course, this is only to be expected since they represent the determinations of a number of observers. Suppose a large number of subjects were to carry out experiments such as those described in the present paper, each finding the same kind of discrepancy between the points X_3 and X'_3 as those of observers A and B, and showing individual differences in the bisection judgments such as those between the readings of A and B. Then if all these results were combined the standard deviations would be considerably increased on account of the wide spreads of the values of X_3 and of those of X'_3. Hence the final graph might well show a considerable overlap between these standard deviations, and so lead to the conclusion that X_3 and X'_3 were identical points within the accuracy of observation. But this conclusion would be wholly misleading.

It is possible to hold the view that "sensation" may be measurable when it is applied to the results given by a number of observers, but that in general it is not measurable when a single individual is concerned. We may thus have to distinguish between the "mean sensation" of the group and the sensation of the individual.

In conclusion, the author wishes to thank the Medical Research Council for grants which have made this work possible, and Dr. J. H. Shaxby for his help and encouragement.

Summary

This paper is analogous to the previous one on hearing. Three discs of white light are simultaneously presented to the eye side by side. The intensity of the middle disc is adjusted until it appears to lie equally in brightness between the brightnesses of the outer discs. Colour and saturation are specifically avoided in this paper in order to make the physical and psychological conditions as simple as possible.

The procedure of experimentation and calculation is entirely similar to the previous paper on sound, and the conclusions drawn are the same; that even under the simplest possible conditions a scale of sensation cannot be constructed.

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The Site of Loss of Water from Insects

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(*Communicated by J. S. Haldane, F.R.S.—Received May* 28, 1934)

INTRODUCTION

A considerable amount of work has been done with regard to the water-balance of insects (recently summarized by Buxton, 1932), and on the physical laws governing the water loss from insects, but as yet no one has determined exactly from what part of the insect's body water is lost. It has been found that when insects are not excreting at all, considerable amounts of water are evaporated from their bodies—quantities frequently sufficient to cause death from desiccation. There are three possible ways in which an insect might lose this water (apart from the alimentary canal) : (i) through the general surface of the body wall; (ii) through the spiracular system; and (iii) partly from the body surface and partly through the spiracular system. The fact that carbon dioxide passes readily through chitin (Dewitz, 1890), and that insects get rid of some of that gas through their integument (v. Buddenbrock and Rohr, 1922), suggests that water-vapour may also pass from the insect's body in a similar manner. However, Hazelhoff (1927) states that resting insects keep their spiracles closed most of the time, only opening them sufficiently often to obtain enough oxygen, in order to conserve water. He believes that most of the water is lost through the tracheal system. The observations of Gunn (1933) on the cockroach and Mellanby (1932, *b*) on the mealworm also suggest that a high proportion of the water evaporated from those insects is lost through the spiracles. The experiments described in this paper show how spiracular opening affects the rate at which insects lose water by evaporation, and the results obtained make it possible to say from what parts of the body this loss takes place.

TECHNIQUE

In most work which has been done on the rate of loss of water from insects, the results have given the *mean* rate of loss over long periods. The water has either been calculated from some measurement of the insect's metabolism, or else it has been collected from batches over long

periods and weighed directly. The difficulty in the way of directly measuring the amount of water produced by a single insect in a short time has been the lack of a method for measuring very small quantities of water under conditions to which insects may be submitted. The simple apparatus described in this paper overcomes the difficulties mentioned

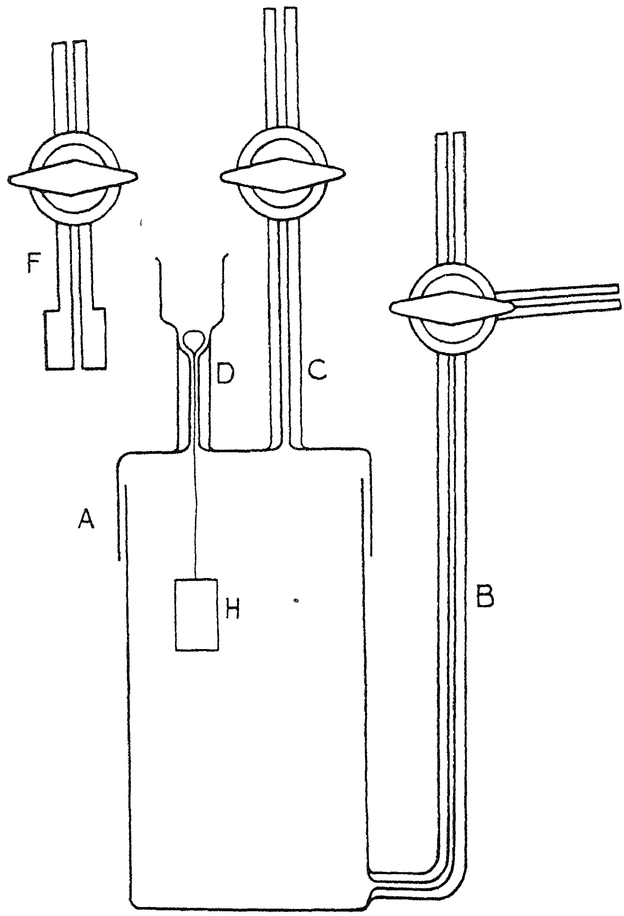

FIG. 1

above, and makes it possible to measure the water evaporated from a single insect in a few hours.

The apparatus, fig. 1, consists of a glass-weighing bottle, of 100 cc internal capacity. The bottle opens at a ground glass joint, A. Three capillary tubes, B, C and D, lead out of the bottle. B leads to a 3-way tap, and C to an ordinary tap. The tube, D, as shown in the figure, broadens out at its distal end into a neck into which a stopper, F, fits.

The bores of B and C are 1 mm and that of D is 2 mm. The stopper, F, is pierced by a capillary which passes through another tap. The humidity inside the apparatus is measured by a paper hygrometer, H, which consists of a small piece of cigarette paper weighing about 15 mg, fastened on to the end of a length of thin copper wire (gauge 42). The wire passes up the capillary tube, D, and ends in a loop at the top end; this loop prevents the hygrometer falling into the bottle. To determine the humidity, the wire loop is raised and connected to the hook of an accurate torsion balance and the paper weighed. The apparatus used gave an accuracy of one hundredth of a milligramme. A description has been given elsewhere (Mellanby, 1933, *b*) of the way in which it is possible to measure humidity with paper hygrometers, but the principle is that paper takes up amounts of water from different atmospheres, which are determined by the relative humidity, irrespective of temperature. Any type of paper can be used, but with the apparatus described above unsized cigarette paper is best, as it quickly comes into equilibrium with any atmosphere. The humidity inside the bottle is determined accurately, because the hygrometer is weighed without being removed from the apparatus, and weighing the hygrometer does not alter the conditions inside the bottle.

To measure the water evaporated from an insect, it was placed inside the bottle, and a quick stream of dry air passed through, from B to D. When sufficient air had passed to drive out all the moisture, the humidity was checked by weighing H. The stopper, F, was then replaced, and all the taps closed. The apparatus was kept at a constant temperature for the period of the experiment (from 2 to 10 hours in different cases), after which the hygrometer was weighed again. In most experiments, the paper increased in weight by 30 to 40 hundredths of a milligramme. Thus the results are accurate to within 3%. The increase in weight of the paper represented part of the water lost by the insect ; the rest was present in the air inside the apparatus. The paper had previously been calibrated, so from its final weight the relative humidity of the air could be determined. As the internal volume of the apparatus (100 cc) and the temperature were both known, the amount of water in the air was easily calculated. The advantage of this method of measuring humidity over any volumetric method is that a small change of temperature does not make the results grossly inaccurate. The principal disadvantage is that the insects are exposed to air, the humidity of which is not constant. However, in these experiments, the humidity of the air never rose above 15%, and in all where results were compared, the conditions were strictly comparable—the humidity at the start of an

experiment was zero, and the length of exposure was adjusted so that the end humidity was always approximately the same.

Tube C was used to introduce mixtures of gases into the apparatus. It was connected to a manometer and a suction pump, and it was possible to remove any fraction of the total air from the apparatus, which was then replaced by the gas required. For instance, to introduce 10% of carbon dioxide, the following procedure was adopted. The taps on B and D were closed, and air sucked out via C until the manometer registered a negative pressure of 7·6 cm. The tap was then closed. A bladder of CO_2 was attached to B, and when the tap was opened the gas rushed into the apparatus to fill it with the correct mixture. The 3-way tap on B made it possible to have the tube full of CO_2 right up to the tap, so that the pure gas was sucked in without any dead space air.

Three species of insects were used in these experiments. They were as follows :—

Mealworms. Larvæ of *Tenebrio molitor* L. Coleoptera.

Clothes moth larvæ. *Tineola biselliella* Hum. Lepidoptera.

Flea larvæ and adults. *Xenopsylla cheopis* Roths. Siphonaptera.

They were selected because previous work (Buxton, 1930 ; Mellanby, 1932, *a*, *b* ; 1933, *a* ; 1934) had determined some essential facts about their metabolism and water-balance, and had shown that they were suitable for use in these experiments. They are also all readily bred in the laboratory.

Results

Previous work on the mealworm (Buxton, 1930 ; Mellanby, 1932, *b*) has shown that the rate at which water is lost during starvation decreases rapidly after tne first few days, and then decreases much more gradually over a period of many weeks. Mealworms were experimented on immediately after removal from their food, and also after starvation in dry air for periods of 14 days and of four months. The mealworms used, when removed from their food, varied between 150 and 200 mg in weight. In all experiments the rate at which they lost water into dry air at 15° C was determined, and also the effect on the water-loss of putting certain mixtures of dry gases into the apparatus. In some experiments a mixture of 99% of nitrogen and 1% of oxygen was used, and in others ordinary dry air plus 5% of carbon dioxide. Both these mixtures have the effect of causing insects to keep their spiracles permanently open (for details, see p. 148).

Results obtained with one individual mealworm immediately after removal from its food are shown in fig. 2. Each black rectangle represents results obtained in one experiment, which lasted for several hours, expressed as the rate of loss of water per hour in one hundredths of a milligramme. As stated above, these figures are accurate to within 3%. The results depicted were obtained at irregular intervals over a period of 14 days, and between each experiment dry air was passed through the apparatus. Sometimes two experiments were made on the same day, while at other times dry air was passed for periods of up to 36 hours before the next reading was taken. The rectangles without any letter at the top represent the results obtained in dry air alone. From these, it

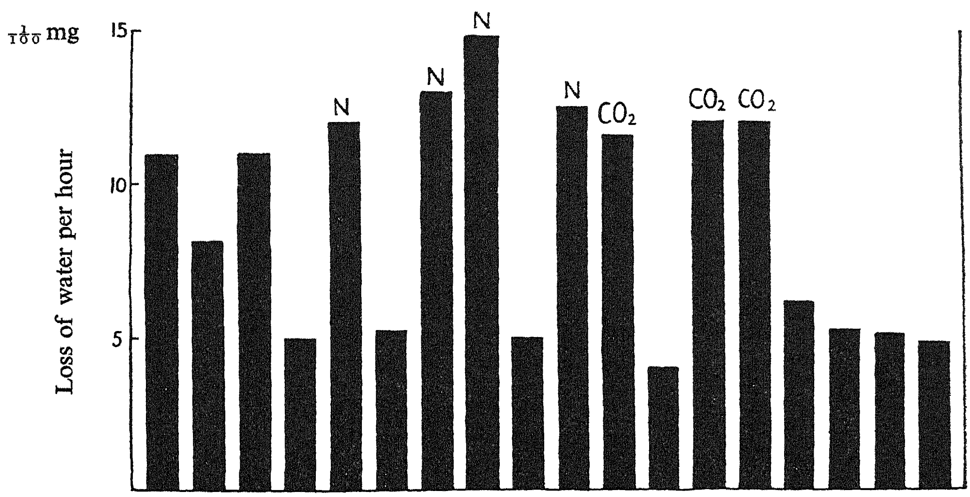

FIG. 2

will be seen that at first the mealworm lost water at a rate of about 11 hundredths of a milligramme per hour, but by the fourth experiment (on the fifth day) the rate had dropped to half that value, from which it did not greatly vary during the next few days. Similar results were obtained using other individuals ; during the first three or four days of starvation the rate of loss of water was much greater and much more irregular than it was subsequently. The effects of the different gases are also shown on fig. 2. The rectangles labelled " N " represent results obtained in 99% nitrogen, and those labelled " CO_2 " represent the results obtained in air containing 5% of carbon dioxide. It will be seen that in all cases these mixtures caused the rate at which water was lost to be increased two- to three-fold—in fact, to rise above the rate at which the unstarved mealworm lost water.

Next, mealworms which had been starved for a fortnight were used. With them, the rate of loss of water in dry air was fairly constant, but it slowly decreased as time went on. The effect of adding 5% of carbon dioxide to the air in the apparatus was to increase the rate of loss of water $2\frac{1}{2}$ times. The average rate of loss, obtained from many observations on several individuals, was 5·1 hundredths of a milligramme per hour into dry air,* and 12·5 hundredths of a milligramme per hour into air containing 5% of carbon dioxide.†

After four months of starvation, the rate at which mealworms lost water into dry air was extremely slow—the average figure was 1·5 hundredths of a milligramme per hour.‡ But when 5% of carbon dioxide was added to the air, the rate of loss of water was increased more than seven times—to 11·1 hundredths of a milligramme per hour.§ It will be seen that this figure is approximately the same as that found for the mealworms starved for only a fortnight. In other words, while starvation greatly decreases the rate at which water is lost into dry air, it hardly, if at all, affects the rate of loss of water into air containing 5% of carbon dioxide. These

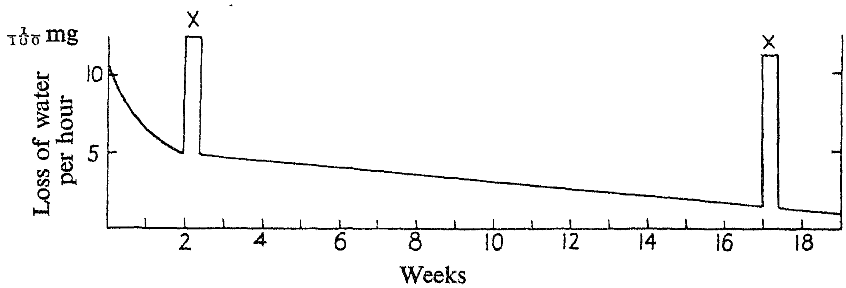

FIG. 3

results are expressed diagrammatically on fig. 3. The curve shows how the rate of loss of water into air decreases as starvation proceeds, and the points marked "X" represent the effects of adding 5% of carbon dioxide to the air.

It was not found practicable to work with individual clothes moth larvæ, because of their small size and because they lost water so slowly. Instead, a batch of 30 individuals was used, with a gross weight of 155 mg. This procedure was not altogether satisfactory, as a few of the larvæ died during the experiment, and this upset the results. (When insects die,

* 32 experiments. P.E. ± 0·04.
† 15 experiments. P.E. ± 0·45.
‡ 20 experiments. P.E. ± 0·1.
§ 12 experiments. P.E. ± 0·6.

their tracheal system fills with water from the tissues, probably due to a change in permeability in the walls of the tracheoles (Wigglesworth, 1931) ; dead insects lose water very rapidly.) As fig. 4 shows, however, the effect of adding 5% of carbon dioxide to the air was to cause an appreciable increase in the rate at which water was lost by the clothes moth larvæ, although the extent of the increase was not always so marked as it was from individual mealworms.

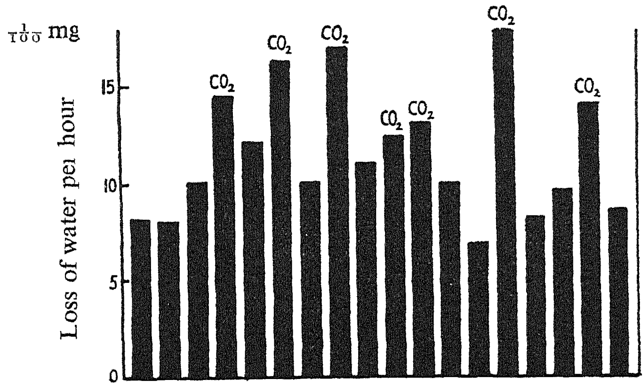

FIG. 4

Previous work (Mellanby, 1932, *a* ; 1933, *a*) on the flea *Xenopsylla cheopis* shows that though the adults are resistant to drying, the larvæ dry up very readily. As the adults have a mechanism for closing the spiracles and the larvæ have none (Sikes, 1930), it was thought that interesting results might be obtained by comparing the rates at which they lose water under various conditions. As described below (p. 147), it is possible to observe the opening and closing of spiracles of the adult flea, and to investigate the effects of gases (CO_2, etc.) on these processes. It was necessary to use batches of fleas and flea larvæ, owing to their very small size.

About 100 " large " flea larvæ were sorted out from the culture, and kept for 12 hours at 23° C, and 90% relative humidity. After this they passed little or no excreta. They were then weighed (total weight = 31 mg), and put into the apparatus. They were exposed to dry air, and to air containing 5% of carbon dioxide. The results obtained are given in fig. 5. The first three experiments were performed within 12 hours, and the remainder on the next day. As these larvæ dry up so readily, it will perhaps be best to consider the first three results only, for whereas all the larvæ would survive 12 hours, some would die after 24. It will be seen that, when the larvæ were exposed to air containing 5% of

carbon dioxide, the rate of loss of water was actually *lower* than in pure dry air. The difference is less than 20%, which may not be significant, but if it is, it probably occurs because the larvæ are active in dry air, but are narcotized by the carbon dioxide. At any rate, it is obvious from all these results that the flea larvæ do not behave in the same manner as do the mealworms or the clothes moth larvæ.

Experiments were next carried out with adults of the same species of flea. About 130 adults (weighing 48 mg) were taken within two days of emerging from their cocoons, and put into a small cage made of phosphor-bronze gauze, which fitted into the bottom of the apparatus. It was

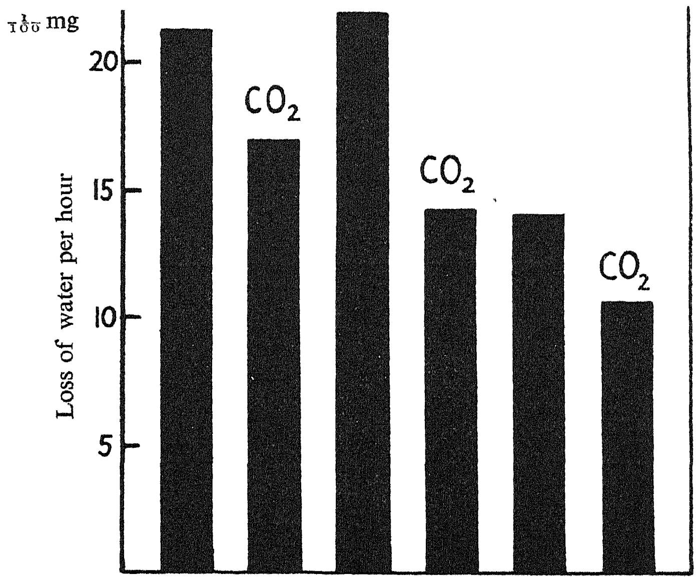

FIG. 5

found necessary to put the adult fleas into this cage, as otherwise they jumped on to the hygrometer and disturbed the weighing. Results obtained are given in fig. 6. The whole experiment only lasted 31 hours, and all the insects were alive and healthy at the end of that time. It will be seen that the adult fleas gave results very similar to those obtained from the mealworms—that is to say, when their spiracles were opened (p. 147), the rate at which water was lost from their bodies by evaporation was more than doubled.

It is interesting to compare the rates at which the adult fleas and flea larvæ lose water. As the weights of the batches used are different, it is necessary to reduce the results to comparable units, and these results

are given in Table 1 in terms of " loss in one hundredths of milligrammes per hour per milligramme of insect."

TABLE 1

Rate at which flea larvæ and adults lose water. Loss expressed as hundredths of a milligramme per hour per milligramme of insect.

	In dry air	In air + 5% CO_2
Flea larvæ	0·69	0·55
Adult fleas	0·28	0·64

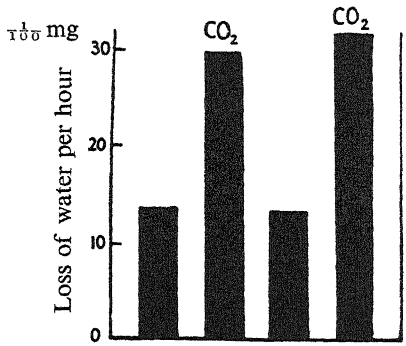

FIG. 6

It will be seen that the rate of loss of water from the adult fleas in dry air is much slower than that from the larvæ, but that in air containing 5% of carbon dioxide the adult flea and the flea larva lose water at much the same rate, and, incidentally, at much the same rate as do the larvæ in pure dry air. The rate at which a mealworm loses water in dry air, expressed in the same units, is about 0·03 one hundredths of a milligramme per milligramme of insect per hour, so even the adult flea is comparatively much less " water-tight."

The next thing determined was the exact proportion of carbon dioxide which caused the insects to open their spiracles permanently. The work of Hazelhoff (1927) and others has shown that carbon dioxide and oxygen lack cause the spiracles to be kept open, but very little exact infotmation exists with regard to the proportion of gases which cause this opening. In a series of experiments which will be published shortly, and to which the author has kindly permitted me to refer, Dr. V. B. Wigglesworth has observed directly and recorded graphically the spiracular movements of the adult flea. In the flea at rest, the spiracles show a rhythmical opening and closing, the duration of opening varying with the temperature and the physiological condition of the insect. He has shown that with a concentration of carbon dioxide of 2% or higher,

the spiracles are kept permanently open, and that the proportion of oxygen must be reduced below 1% to have the same effect.

Experiments made on the rate of loss of water from the mealworm in different mixtures of gases confirm the results mentioned above. The rate of loss from individuals starved for a fortnight was at least twice the normal when more than 2% of carbon dioxide was added to the air in the apparatus, and it was necessary to reduce the percentage of oxygen below 1% before the rate of loss of water was appreciably increased. Lower concentration of carbon dioxide and slightly higher concentrations of oxygen gave very irregular results.

Discussion

During rest, those insects which are able to close their spiracles keep them closed during the greater part of the time (Hazelhoff, 1927), and the amount they are opened depends principally on the rate of metabolism of the insect. In the fasting mealworm, as starvation proceeds, the rate of metabolism gradually decreases (Buxton, 1930). At the same time, the rate at which water is lost by evaporation from the insect also decreases. When, however, the spiracles of the mealworm are permanently open, the rate at which water is evaporated increases greatly, and this increase is independent of the metabolic rate. This can only mean that practically all the water which is evaporated from the insect's body is evaporated through the tracheal system. This conclusion is supported by the fact that two independent methods which cause the insects to open their spiracles—excess carbon dioxide and lack of oxygen—have the same influence on the rate of water loss. Also as carbon dioxide does not affect the water loss from the flea larva, which cannot regulate its spiracular opening, the increased evaporation from the mealworm cannot be due to some specific action of that gas.

It has been shown that the flea larva and adult both lose water at the same rate when they have their spiracles permanently open. Now the larva has an extremely thin cuticle, while that of the adult is comparatively thick. This result must mean that both cuticles, independent of their thickness, are equally water-tight. Therefore insects inhabiting dry environments require effective spiracle-closing mechanisms, and not thick sclerotized cuticles.

I am indebted to Professor P. A. Buxton and to Dr. V. B. Wigglesworth for their helpful criticisms.

Summary

An apparatus is described which will measure the amount of water evaporated from an insect, accurate to one hundredth of a milligramme.

The rate of loss of water from three species of insect was determined, first in dry air, then in air to which 5% of carbon dioxide had been added, and thirdly in a mixture containing under 1% of oxygen. In insects with a spiracle-closing mechanism, the rate of loss of water under the second two conditions (which caused them to keep their spiracles open permanently) was two to seven times that in dry air. In insects which could not close their spiracles, the rate of loss of water was practically the same under all conditions.

It was found that 2% of carbon dioxide in the air was sufficient to cause insects to keep their spiracles permanently open, and that the oxygen had to be reduced below 1% to have the same effect.

From these experiments, it appears that practically all the water evaporated from an insect is lost by way of the tracheal system, and that a thin integument may be just as water-tight as one which is highly "sclerotized."

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Researches on Plant Respiration

III—The Relationship between the Respiration in Air and in Nitrogen of certain Seeds during Germination (a) Seeds in which Fats constitute the chief Food Reserve

By William Leach, D.Sc., and Kenneth W. Dent, B.Sc.

(*Communicated by W. Stiles, F.R.S.—Received May* 31, 1934)

Introduction

The relationship between aerobic and anærobic respiration has for some years been a subject which has interested plant physiologists, and as early as 1885 Pfeffer published values obtained in his laboratory for the ratios of the respiration intensities in hydrogen to those in air in the case of seedlings, fruits, and shoots of various species of plants. In 1913 Hill gave an account of investigations on respiration rates in air, nitrogen, and hydrogen using as his experimental material certain ripe and unripe fruits and also germinating wheat. The work of Flieg, published in 1922, is of a somewhat similar type to that here described ; Flieg obtained the changes in the respiration rate, and also the changes in the respiratory quotient, of *Aspergillus niger* when cultures of this organism were successively kept in atmospheres of air, nitrogen, and again air. Boysen Jensen in 1923 described experiments similar to those of Hill but carried out with *Aspergillus* grown on prune extract, and also with leaves of *Syringa vulgaris*, *Tropæolum majus*, and seedlings of *Sinapis alba* ; in these experiments, however, hydrogen instead of nitrogen was used as the anærobic atmosphere. A critical series of investigations into the subject of the relative effects of air and nitrogen upon the respiration of apples has been described by Blackman and Parija in 1928. From an analysis of the results of these investigations Blackman has elaborated a theoretical scheme to explain the respiratory process in the light of his experimental data ; to these papers we would refer our readers.

The investigations described in the present paper, dealing with the respiration in air and in nitrogen of certain fat-storing seeds during germination were undertaken in order to see how far Blackman's scheme can be applied to respiring tissues other than those he used. They were

also carried out for the purpose of finally confirming the suitability of the katharometer method for work of this kind ; consequently with *Helianthus annuus* and *Ricinus communis* duplicate sets of experiments were performed in which the respiration intensities, as indicated by carbon dioxide output, were measured by the Pettenkofer method and by the katharometer respectively. It will be seen from the results of these experiments that the katharometer method is very suitable for such work, and, in fact, possesses a number of distinct advantages over the older method.

Material and Methods

The species used in these investigations are given below together with the sources of the seeds and the varieties used. In each case fat forms the predominant food reserve.

Ricinus communis major (Sutton).
Helianthus annuus (Simpson's Giant Single, and Sutton's Giant Yellow Sunflower).
Cucurbita pepo (Sutton's Long White Vegetable Marrow).

As already stated the experimental data to be described in this paper were obtained by both the katharometer and the Pettenkofer methods. The katharometer method used was the same as that used and described in Part II (Stiles and Leach, 1933), automatic records being obtained of both the increase in carbon dioxide, and the change in pressure within the closed experimental plant chamber ; in most experiments single seeds were used.

In the Pettenkofer method the experimental seedlings (twelve of *Ricinus* and thirty of *Helianthus*) were kept on wet cotton wool supported on a glass tripod standing in the glass respiration chamber. This respiration chamber was composed of two hemispherical halves ground together, and was immersed in a large constant temperature bath thermostatically maintained at a temperature of 25° C. Air (or nitrogen) previously freed from carbon dioxide and saturated with moisture was passed into the respiration chamber at the top and conducted out at the bottom whence it passed through a glass tube to a distributor tube having sixteen outlets connected with the inlet ends of the sixteen Pettenkofer tubes containing standard baryta solution. The outlet ends of the latter were connected to a Blackman air current commutator, by means of which each of the Pettenkofer tubes was successively switched into the gas circuit for a period of three hours, thus enabling continuous records of respiration to be obtained. The outlet from the commutator

was connected to a filter pump which produced the necessary current through the apparatus when air was being used. The nitrogen used for the anærobic experimental periods was obtained from commercial cylinders, the gas being passed through alkaline pyrogallol solution to rid it of any trace of oxygen that may have been present. Analysis by means of the Haldane gas analysis apparatus showed that the nitrogen thus treated contained no measurable amount of oxygen. This nitrogen supply was connected by means of a two-way tap to the inlet of the respiration chamber. The outlet of the chamber was also fitted with a similar tap to enable a rapid stream of gas to be passed through it for scavenging purposes. When respiration in nitrogen was being measured it was naturally not found necessary to use the filter pump, the gas pressure in the cylinder maintaining the current through the apparatus.

In all the experiments here described the carbon dioxide output of the experimental material is expressed in milligrams per gramme fresh (unsoaked) weight of seed (without seed coat) per hour, and the seedlings were maintained throughout the periods of experimentation at a temperature of 25° C.

Experimental Results

1 *Helianthus Annuus*

The normal course of respiration in air of a single seed of Helianthus during germination, and the accompanying changes in the value of the respiratory quotient, are shown in fig. 1 (see experiment 98, Stiles and

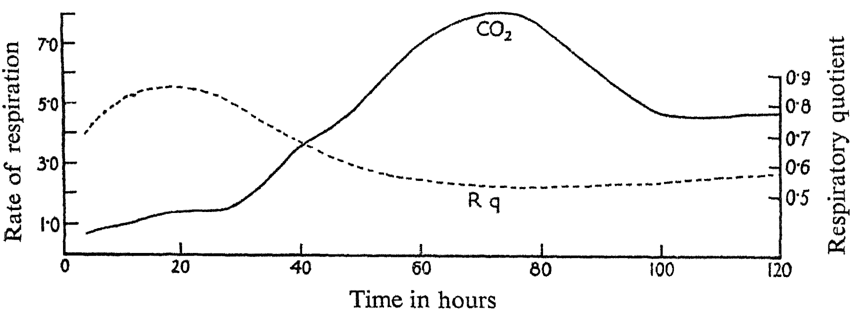

Fig. 1—*Helianthus*, experiment 98

Leach, 1933). The changes in respiration intensity and respiratory quotient values shown by germinating seeds of this species when alternately surrounded by air, an atmosphere of nitrogen, and finally air, respectively, are shown in Tables I and II, and figs. 3 and 4. Table II and fig. 2 show respiration rates as determined by the Pettenkofer method

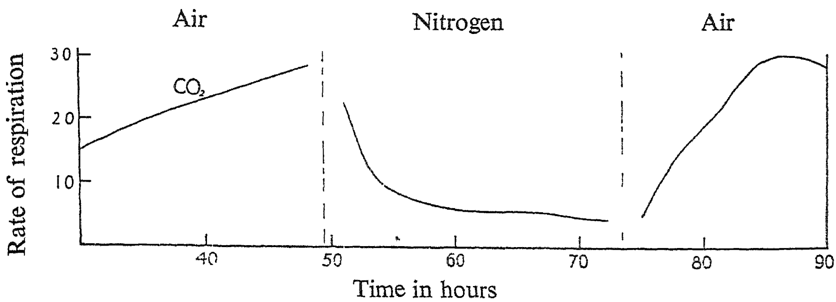

FIG. 2—*Helianthus*, experiment A 15

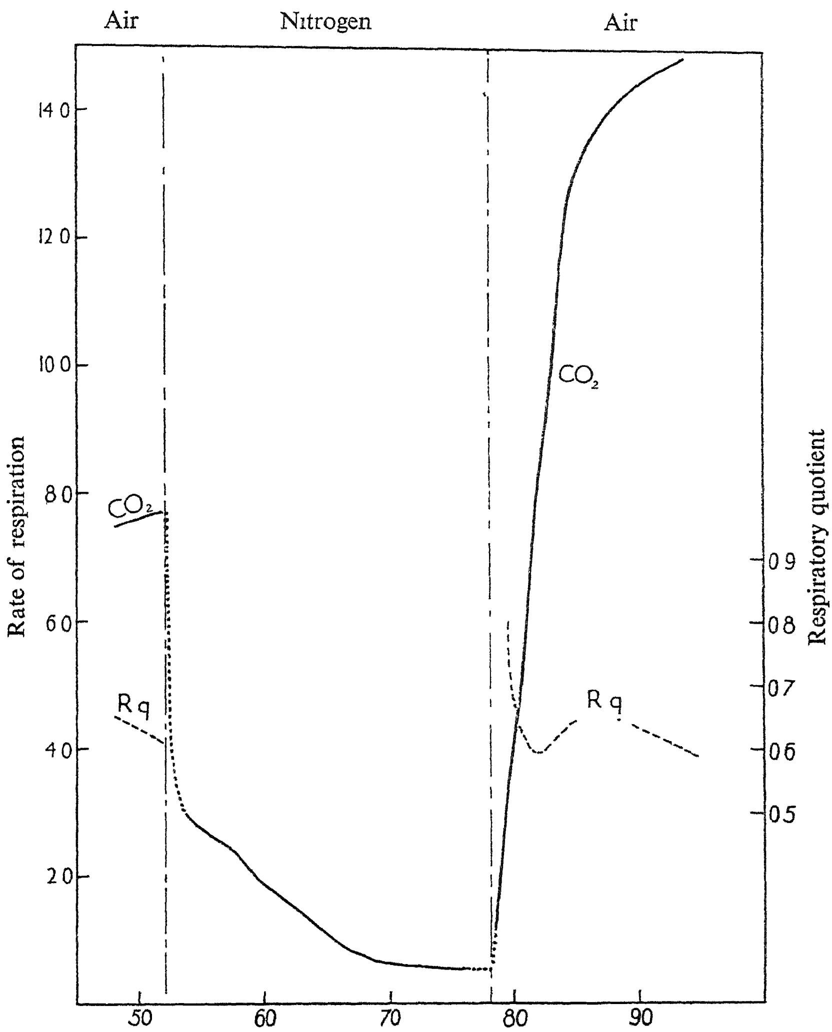

FIG. 3—*Helianthus*, experiment 196

while Table I and figs. 3 and 4 give data of both respiration rate and respiratory quotient as determined by the katharometer and the recording manometer. With regard to the respiration rates, certain differences exist between the results obtained by the two methods. It will first be observed that the respiration intensities recorded by the katharometer experiments are higher than those obtained with the Pettenkofer apparatus. This is due to the fact that seeds of Sutton's "Giant Yellow" sunflower were used in the former, whereas those of Sutton's "Giant

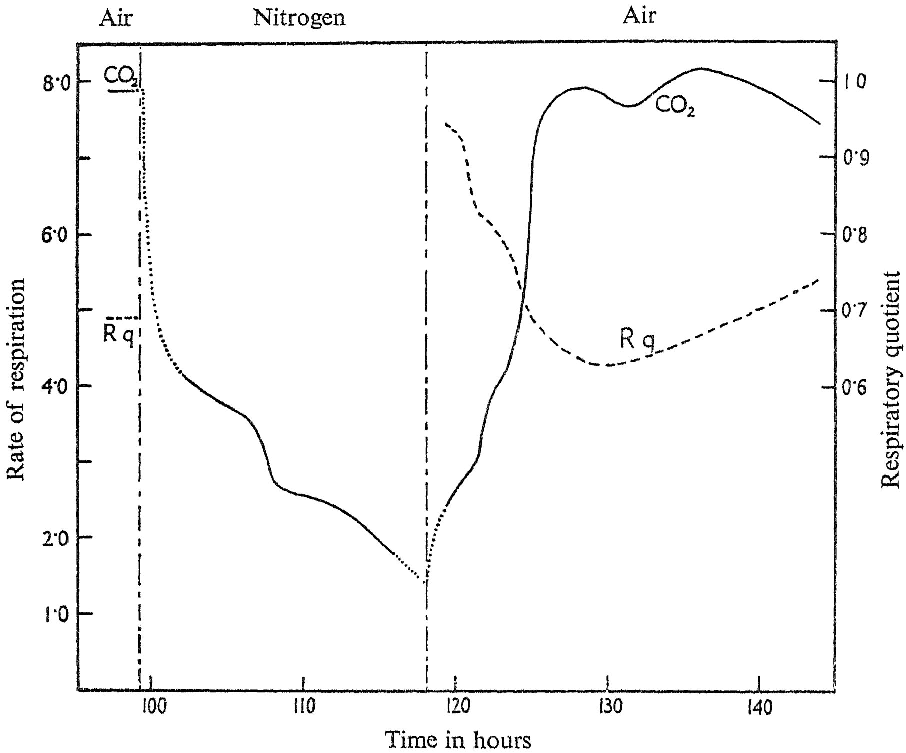

FIG. 4—*Helianthus*, experiment 195

Single" variety were used with the latter. Another difference is shown in the change in respiration rate which results from a replacement of an atmosphere of air by one of pure nitrogen. It will be noted that the katharometer records a very rapid initial fall in the carbon dioxide output when this change takes place, whereas the Pettenkofer method, in every experiment, shows a much more gradual fall. This must be attributed to a time lag resulting from a certain lack of sensitivity of the latter method and would appear to be due to the relatively long time taken to

Table I—The respiratory behaviour of *Helianthus annuus* seedlings in air and in nitrogen. (Katharometer experiments)

Experiment number

172			193			196			194			195		
Age of seedling in hours	Respiration rate mg CO_2 per gm per hour	Rq	Age of seedling in hours	Respiration rate mg CO_2 per gm per hour	Rq	Age of seedling in hours	Respiration rate mg CO_2 per gm per hour	Rq	Age of seedling in hours	Respiration rate mg CO_2 per gm per hour	Rq	Age of seedling in hours	Respiration rate mg CO_2 per gm per hour	Rq
	Air			Air			Air			Air			Air	
72·5	7·50	0·62	49·5	5·20	0·78	49·0	7·53	0·64	123·0	9·50	0·64	98·0	7·89	0·69
73·0	Nitrogen	—	51·0	Nitrogen	—	51·0	7·63	0·62	124·0	Nitrogen	—	99·25	Nitrogen	—
75·0	3·57	—	53·0	1·70	—	52·0	Nitrogen	—	125·5	4·83	—	102·0	4·14	—
78·0	2·58	—	55·0	1·22	—	54·5	2·78	—	127·5	3·26	—	104·0	3·89	—
81·0	2·06	—	57·0	1·17	—	57·5	2·40	—	129·5	2·68	—	106·0	3·65	—
84·0	1·83	—	64·0	0·73	—	59·5	1·92	—	131·5	2·43	—	108·0	2·78	—
87·0	1·44	—	72·5	0·54	—	62·5	1·51	—	133·5	2·43	—	112·0	2·43	—
90·0	1·01	—	75·0	Air	—	69·0	0·64	—	136·0	2·43	—	116·0	1·80	—
93·0	1·41	—	76·5	3·02	0·64	76·0	0·54	—	138·5	1·95	—	118·25	Air	—
98·0	1·69	—	79·5	3·89	0·70	78·0	Air	—	140·0	Air	—	119·5	2·43	0·94
101·5	1·58	—	84·5	4·23	0·65	79·5	3·52	0·80	141·0	1·95	0·84	120·5	2·78	0·92
105·5	1·26	—	87·5	4·62	0·64	80·5	4·97	0·63	142·0	2·43	0·75	121·5	3·02	0·83
109·5	1·21	—	89·5	5·20	—	81·5	7·21	0·60	143·0	5·36	0·74	122·5	3·89	0·81
112·5	Air	—	92·0	6·20	—	82·5	9·13	0·60	144·5	6·19	0·58	123·5	4·28	0·78
113·5	3·81	0·75	96·5	7·06	0·61	83·5	10·90	0·62	148·5	8·04	0·58	125·0	6·68	0·69
114·5	4·51	0·71	98·5	7·06	0·59	84·5	12·82	0·64	150·0	8·29	0·59	127·0	7·80	0·65
115·5	5·50	0·74	—	—	—	93·5	14·89	0·60	152·5	8·32	—	129·0	7·89	0·63
116·5	5·99	0·76	—	—	—	—	—	—	157·5	8·09	—	131·0	7·65	0·63
									161·0	7·79	—	133·0	7·80	—
									162·5	7·39	0·69	136·0	8·14	—
												144·0	7·45	0·74

Table II—The respiratory behaviour of *Helianthus annuus* seedlings in air and in nitrogen

Pettenkofer experiments

Experiment A 15: Age of seedling in hours	A 15: Respiration rate mg CO_2 per gm per hour	A 17: Age of seedling in hours	A 17: Respiration rate mg CO_2 per gm per hour	A 18: Age of seedling in hours	A 18: Respiration rate mg CO_2 per gm per hour	A 21: Age of seedling in hours	A 21: Respiration rate mg CO_2 per gm per hour	A 22: Age of seedling in hours	A 22: Respiration rate mg CO_2 per gm per hour
	Air		Air		Air		Air		Air
27	1·33	97	2·98	117	3·78	9	0·48	6	0·25
30	1·21	100	3·62	120	3·78	12	0·48	9	0·37
33	1·82	103	4·90	123	3·91	15	0·96	12	0·37
36	2·27	106	4·67	126	3·53	18	0·96	15	0·49
39	2·27	109	4·03	129	4·43	21	1·56	18	0·62
42	2·41	112	3·62	132	4·97	24	1·56	21	0·86
45	2·58	115	3·82	135	3·53	25·5	Nitrogen	22·5	Nitrogen
48	2·87	118	4·03	138	3·13	27	1·20	24	0·86
49·5	Nitrogen	119·5	Nitrogen	141	2·87	30	0·60	27	0·62
51	2·27	121	3·40	144	3·65	33	0·48	30	0·37
54	1·06	124	2·13	145·5	Nitrogen	36	0·42	33	0·37
57	0·76	127	1·70	147	3·65	39	0·36	36	0·31
60	0·60	130	1·49	150	1·83	42	0·30	39	0·25
63	0·60	133	1·28	153	1·53	45	0·24	42	0·18
66	0·60	136	1·28	156	1·31	48·	0·24	45	0·18
69	0·45	139	1·06	159	1·31	49·5	Air	46·5	Air
72	0·45	142	1·06	162	1·31	51	1·80	48	0·62
73·5	Air	143·5	Air	165	1·05	54	1·80	51	1·11
75	0·45	145	1·49	168	1·05	57	3·73	54	1·47
78	1·51	148	2·67	169·5	Air	60	3·73	57	1·84
81	2·12	151	4·67	171	1·96	63	4·44	60	2·22
84	2·87	154	4·47	174	1·96	66	4·44	63	2·67
87	3·03	157	4·88	177	1·96	—	—	66	1·97
90	2·87	160	3·82	180	1·96				
93	2·72	—	—	183	3·27				
96	2·58	—	—	186	3·27				
				189	4·25				
				192	4·25				

effect a complete removal of oxygen from the respiration chamber when changing over to an atmosphere of nitrogen. It must be remembered also that in the katharometer experiments single seeds were used whereas in the Pettenkofer experiments batches of thirty seeds were used in each case, so that in the latter we are dealing with the average behaviour of populations of seedlings.

If we examine the respiratory behaviour of *Helianthus* seedlings, when transferred from an atmosphere of air to one of nitrogen we find that coincident with the change in the external gas is a marked fall in the rate of carbon dioxide output by the seedlings. Following on this initial rapid decrease, the respiration rate falls off more slowly and may finally attain an almost level value. Experiments A 15 to A 22 show this latter falling off to be very regular, but the katharometer experiments indicate that it may not be so regular if the behaviour of individual seedlings, instead of that of batches of seedlings as a whole, is examined. The return to normal aerobic conditions causes an increase in the respiration rate, which may continue to rise to a maximum value and then again to fall.

If the behaviour of the respiratory quotient is now examined in relation to the changes in carbon dioxide output described above, some interesting facts come to light. The quotient values of the seedlings at the beginnings of the experiments will be seen, on comparison with corresponding values shown in fig. 1 and those given in Part II (Stiles and Leach, 1933), to lie within the limits of variability exhibited by different seedlings. It will be observed that immediately after the conditions are changed from anærobic to aerobic, the respiratory quotient shows a falling course for a few hours after which it again begins to rise. This latter rise may continue as in experiments 194 and 195, or it may, after a short time, be again followed by a fall as in experiments 193 and 196.

A more detailed examination of these data regarding the respiratory behaviour of sunflower seedlings in air and nitrogen brings out certain significant points. In the first place the very rapid fall in respiration rate, at the onset of anærobic conditions, to values varying from approximately half of the aerobic value to much less, would appear to be due not to an immediate reduction in the amount of available substrate, but to the change over from the post-glycolytic, complete oxidation stage of the aerobic respiratory process, to the anærobic breakdown, which involves the liberation of carbon dioxide and the probable formation of alcohol. It further would appear from the experimental results obtained that this latter process may continue for a considerable number of hours without causing permanent injury to the respiring cells.

After undergoing an experimental period of anærobiosis in nitrogen, if the seedlings are brought into air, the respiration rate rises until it again follows the course found to be typical of germinating *Helianthus* seedlings under aerobic conditions. As already mentioned the experiments with the katharometer indicate that this return to the normal " air line " respiration takes place at a comparatively rapid rate. The subsequent falling off in the rate of carbon dioxide output shown in experiment A 17 would appear to be due to the fact that the seedlings used were in a relatively advanced stage of development although it may in part be due to desiccation. This latter was certainly responsible for the falling off in respiration rate in experiment A 15 after the eighty-seventh hour from the beginning of the germination of the seeds. In this connection it may be mentioned that it was found that prolonged maintenance of the experimental seedlings in a continuous gas stream always resulted in the apparent drying up of their root tips, thus causing them to turn brown and die, and further, this injury occurred in spite of the precautions taken to saturate the incoming gas with water vapour.

The changes in the value of the respiratory quotient after the seedlings are transferred from nitrogen to air are significant. These changes have already been referred to and it would appear that the developmental stage of the seedling has a considerable influence on behaviour of the respiratory quotient immediately after a period of anærobiosis. In experiments 172, 193, and 196 the seedlings were subjected to the period in nitrogen at an earlier stage of development than in experiments 194 and 195. It will be observed that for the former the initial fall in the value of the quotient is more rapid and of shorter duration than it is for the latter. Further, whereas in experiments 193, and 196 this initial fall in the quotient value is followed by a rise and a subsequent second fall, in experiments 194 and 195 this second fall is absent during the period of experimentation used in these investigations. As this influence of development on respiratory behaviour forms a definite problem requiring detailed examination, it is proposed not to deal further with it here but to leave it for consideration in a later paper.

2—*Cucurbita Pepo*

The seed of *Cucurbita* is non-endospermic and morphologically similar to that of *Helianthus*, its epigeal germination is also of the same type. The normal course of respiration of a single *Cucurbita* seedling, and the changes that take place in the value of the respiratory quotient, when the

seedling is kept in the katharometer plant chamber and maintained at a temperature of 25° C are shown in fig. 5. From this figure it will be seen that after about forty hours from the beginning of germination, the respiration rate rises for a time with considerable rapidity, while the respiratory quotient shows a steadily falling value.

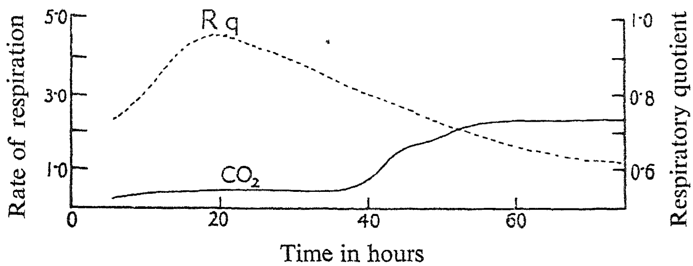

FIG. 5—*Cucurbita*, experiment 155

The three experiments here described which furnish data of the respiratory behaviour of seedlings of *Cucurbita* when alternately subjected to atmospheres of air, nitrogen, and air, were all carried out with the katharometer apparatus. The results of these experiments are numerically given in Table III. An examination of the numbers given in this table shows that in all three experiments the seedlings behaved in a very similar manner both as regards the courses of their respiration during the experimental period and as regards the changes in the values of their respiratory quotients. This similarity must largely be due to the fact that all the seedlings used were in approximately corresponding developmental stages, the respiration data in each case being obtained over the period between the thirtieth and the hundredth hour from the beginning of germination.

The changes in respiratory behaviour which result when a *Cucurbita* seedling is successively brought into an atmosphere of air, of nitrogen, and finally of air, are indicated graphically in fig. 6. It will be noted that in a general way the changes in the external gas produce in *Cucurbita* seedlings similar effects on the rate of carbon dioxide evolution as have already been described as being produced in *Helianthus* seedlings. During the first experimental period in air the carbon dioxide output and the respiratory quotient follow the normal developmental courses as already described above. The change from an atmosphere of air to one of nitrogen produces a sudden fall in the respiration rate which in *Cucurbita* after about three hours from the time when the atmosphere is changed to nitrogen stands at approximately one-third of what its rate was in air

Table III—The respiratory behaviour of *Cucurbita pepo* seedlings in air and in nitrogen

(Katharometer experiments)

Experiment 179			Experiment 187			Experiment 190		
Age of seedling in hours	Respiration rate mg CO_2 per gm per hour	Rq	Age of seedling in hours	Respiration rate mg CO_2 per gm per hour	Rq	Age of seedling in hours	Respiration rate mg CO_2 per gm per hour	Rq
	Air			Air			Air	
49	2·48	0·68	40·5	1·96	0·85	25·75	2·34	0·87
51·5	Nitrogen	—	45·5	2·32	0·78	29·75	2·40	0·89
54·0	0·81	—	48·5	2·49	0·77	33·75	2·18	0·87
56·0	0·70	—	50·0	Ntirogen	—	37·75	2·48	0·87
60·0	0·63	—	51·5	0·95	—	41·25	2·81	—
66·5	0·56	—	53·0	0·75	—	41·50	Nitrogen	—
70·5	Air	—	55·0	0·74	—	44·25	1·36	—
72·5	1·82	0·47	59·0	0·61	—	45·25	0·94	—
74·5	1·97	0·61	66·5	0·46	—	48·25	0·67	—
94·5	4·24	0·47	68·0	Air	—	51·75	0·81	—
			69·0	1·02	0·64	55·75	0·81	—
			70·5	2·04	0·68	59·75	0·69	—
			72·5	2·19	0·70	63·25	0·36	—
			92·5	4·62	0·61	65·00	Air	—
						66·25	2·65	0·92
						67·25	2·65	0·61
						68·25	2·81	0·68
						70·75	3·12	—
						72·75	3·59	—
						91·25	4·99	0·67

immediately before the change was made. From this point onwards until the nitrogen is again replaced by air the three experiments performed indicate that the respiration rate gradually but definitely falls off. In experiment 190 this falling off is more rapid than in the other two experiments; in fact, the results of experiment 179 suggest that after the sixtieth hour an approximately constant rate of carbon dioxide output was reached its value being about 0·23 of the rate before the change from air to nitrogen took place. The subsequent change from nitrogen to air brings about a very rapid rise in the rate of carbon dioxide evolution until it stands a little lower than it did immediately before the beginning of the period in nitrogen. The rate then rises steadily and at a fairly rapid rate during the remainder of the experimental period.

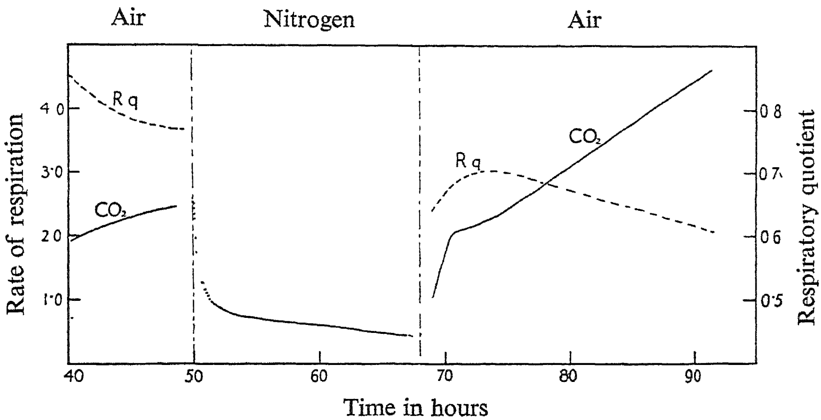

FIG. 6—*Cucurbita*, experiment 187

The respiratory quotient, after the final change from nitrogen to air, differs somewhat in its behaviour from what happens in the case of *Helianthus*. Shortly after the change from nitrogen to air has been made the respiratory quotient is considerably lower than it was immediately previous to the first change from air to nitrogen. From this low value it rapidly rises until after some five to ten hours it reaches a value approximating to that at which it stood before the change from air to nitrogen was made. It then appears again to take up the downward drift that we have seen is characteristic of the normal developmental respiratory behaviour.

An interesting feature similar to that noted in connection with *Helianthus* and discussed further on p. 168 is shown in experiment 190. In this seedling the first hour after the change from nitrogen to air is made is characterized by a rapidly falling respiratory quotient value.

3—*Ricinus Communis*

The seed of *Ricinus* differs from those of *Helianthus* and *Cucurbita* in that it is endospermic whereas the latter are non-endospermic. This morphological feature must be borne in mind when the respiratory behaviour of the germinating *Ricinus* seed is being considered. It is to be supposed that the cotyledons, during germination, are the seat of active respiration and it may well be that this cotyledonary respiration is affected as a result of these organs being embedded in the compact mass of endosperm tissue which must considerably restrict the diffusion of oxygen and carbon dioxide.

The respiration measurements carried out with this species were made, as for *Helianthus*, both with the continuous current Pettenkofer apparatus and also in the still atmosphere of the closed chamber of the katharometer. Again, as will be seen from the numbers given in Tables IV and V, the values obtained by the two methods of experimentation correspond to a degree which is well within the limits of variability shown by the behaviour of different individual seedlings. With *Ricinus*, the already-mentioned lack of sensitivity of the Pettenkofer apparatus as compared with the katharometer is again evident so that measurements obtained with the former often fail to indicate the sudden and marked changes in the rate of carbon dioxide output that are shown by the latter when the gas surrounding the respiring seedlings is changed from air to nitrogen and *vice versa*.

If the numbers given in Tables IV and V, which denote the rates of evolution of carbon dioxide in *Ricinus* seedlings in air and nitrogen, are examined and compared with corresponding ones given in Tables I, II, and III for the two species already dealt with, it will be seen that, in the main, the experimental conditions produce similar effects on the respiration of *Ricinus* seedlings as they do on the respiration of those of *Helianthus* and *Cucurbita*, so far as carbon dioxide output is concerned.

Transferring the seedling from air to nitrogen, as shown by the katharometer, results in a sudden reduction in the rate of carbon dioxide output to between one-half and one-third of what it stood at in air. Continued respiration in nitrogen is characterized by a very definite and steady fall in the rate of carbon dioxide output until, as indicated in experiments 184 and 185, respiration after a time may even cease, or presumably fall to an extremely low rate. It would appear that the time taken for the respiration to be reduced to this negligible rate depends upon the metabolic activity of the seedling. For example, in experiment 184, fig. 7, where immediately before the change from air to nitrogen was made,

Table IV—The respiratory behaviour of *Ricinus communis* seedlings in air and in nitrogen

(Katharometer experiments)

Experiment

184

Age of seedling in hours	Respiration rate mg CO_2 per gm per hour	Rq
	Air	
53·0	2·43	0·73
54·5	Nitrogen	—
56·0	0·91	—
58·0	0·82	—
60·0	0·70	—
62·0	0·61	—
66·0	0·42	—
73·5	0·16	—
77·25	Air	—
79·0	1·56	0·65
81·0	2·00	0·61
83·0	2·61	0·64
85·0	2·77	—
91·0	3·52	—
96·5	4·54	0·63

185

Age of seedling in hours	Respiration rate mg CO_2 per gm per hour	Rq
	Air	
26·25	0·76	0·51
28·5	Nitrogen	—
30·5	0·32	—
33·5	0·32	—
36·5	0·27	—
42·5	0·25	—
53·0	0·14	—
63·5	0·14	—
81·0	0·05	—
90·5	Air	—
93·5	0·63	0·77
98·5	0·67	0·69
103·5	0 86	0·77
108·5	1·04	0·77
112·5	1·35	—
118·5	1·41	0·77
121·0	1·47	0·71

198

Age of seedling in hours	Respiration rate mg CO_2 per gm per hour	Rq
	Air	
46·5	2·20	0·83
49·5	2·30	0·82
50·75	Nitrogen	—
52·5	1·45	—
54·5	1·13	—
56·5	0·91	—
58·5	0·79	—
60·5	0·74	—
62·5	0·67	—
65·0	Air	—
66·5	1·21	0·75
68·5	1·63	9·71
70·5	2·23	0·71
72·5	2·55	0·72
77·5	3·93	0·71
80·5	4·29	0·70
84·5	5·03	0·69

Table V—The respiratory behaviour of *Ricinus communis* seedlings in air and in nitrogen

(Pettenkofer experiments)

Experiment A 5: Age of seedlings in hours	A 5: Respiration rate mg CO_2 per gm per hour	A 8: Age of seedlings in hours	A 8: Respiration rate mg CO_2 per gm per hour	A 10: Age of seedlings in hours	A 10: Respiration rate mg CO_2 per gm per hour	A 11: Age of seedlings in hours	A 11: Respiration rate mg CO_2 per gm per hour
	Air		Air		Air		Air
124·0	0·94	51·0	0·80	60·0	1·39	51·0	0·69
127·0	1·63	54·0	0·93	63·0	1·57	54·0	2·25
128·5	Nitrogen	57·0	1·30	66·0	1·57	55·5	Nitrogen
130·0	1·88	60·0	1·23	69·0	1·63	57·0	1·43
133·0	1·44	63·0	1·30	72·0	1·87	60·0	1·00
136·0	1·00	66·0	1·37	75·0	2·06	61·0	0·81
139·0	0·82	69·0	1·48	76·5	Nitrogen	63·0	0·63
142·0	0·63	72·0	1·55	78·0	1·21	66·0	0·44
145·0	0·50	73·5	Nitrogen	81·0	0·85	69·0	0·31
148·0	0·50	75·0	1·17	84·0	0·73	72·0	0·31
149·5	Air	78·0	0·93	87·0	0·54	75·0	0·25
151·0	0·94	81·0	0·74	90·0	0·48	78·0	0·25
154·0	0·94	84·0	0·62	93·0	0·42	79·5	Air
157·0	1·07	87·0	0·56	96·0	0·42	81·0	0·75
160·0	1·28	90·0	0·43	97·5	Air	84·0	0·81
163·0	1·38	93·0	0·43	99·0	0·30	87·0	1·43
166·0	1·38	96·0	0·37	102·0	0·67	90·0	1·56
169·0	1·13	97·5	Air	105·0	0·97	93·0	1·62
172·0	1·88	99·0	0·37	108·0	1·21	96·0	1·62
		102·0	0·73	111·0	1·27	99·0	1·68
		105·0	0·93	114·0	1·09		
		108·0	1·17	117·0	1·15		
		111·0	1·37	120·0	1·33		
		114·0	1·48	123·0	1·87		
		117·0	1·61	126·0	1·51		
				129·0	1·76		

carbon dioxide was being evolved at the rate of 2·43 mg per gram per hour, a period of 22·75 hours in nitrogen resulted in a fall in carbon dioxide output to 0·16 mg per gram per hour. On the other hand, the seedling used in experiment 185 was in an early stage of germination immediately before its transference from air to nitrogen, and was giving out carbon dioxide at the rate of only 0·76 mg per gram per hour. This latter seedling required a period of 52·5 hours in nitrogen to bring about a reduction of its respiration rate to 0·09 mg carbon dioxide per gram per hour.

These results would appear definitely to indicate that the gradual falling off in the respiration rate that takes place when the seedlings are

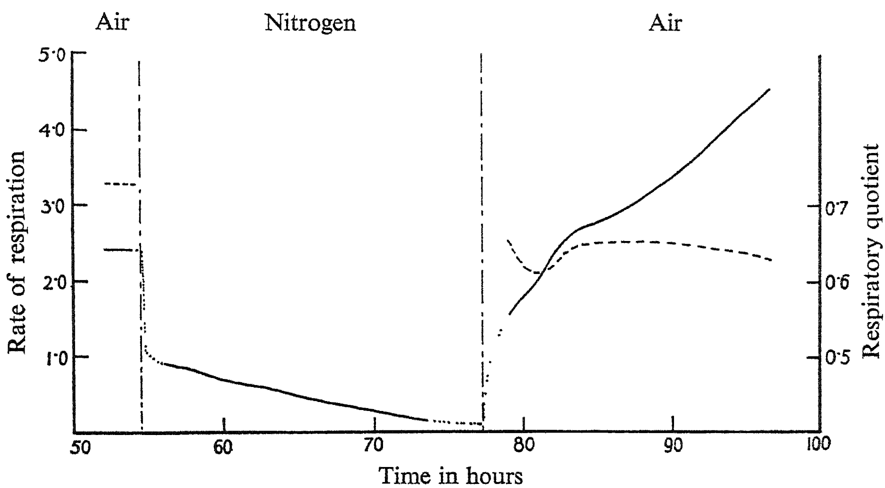

FIG. 7—*Ricinus*, experiment 184

in nitrogen is due to a gradual using up of the available respirable substrate, and the rate at which the falling off takes place depends upon the enzyme activity at the time when anærobiosis takes place.

The behaviour of the respiratory quotient for *Ricinus*, when a seedling is transferred to air after a period in nitrogen, corresponds closely with what has been described above for *Helianthus* seedlings. The normal course of the respiratory quotient in relation to the rate of carbon dioxide output during the development of a seedling of this species is shown in fig. 8. It will be noted that while the carbon dioxide output shows a steady increase throughout the experimental period, the quotient, after the fifteenth hour from the beginning of germination, undergoes a corresponding almost continuous decrease in value.*

* See Stiles and Leach, 1933, experiment 107, p. 419.

If fig. 7 and Table IV are examined it will be noted that, after the seedlings are transferred from nitrogen to air, the respiratory quotient is first of all falling rapidly. After this fall the quotient undergoes an equally rapid rise which continues for a few hours subsequent to which it again begins to fall. This final falling course would appear to owe its origin to the fact that the respiratory activities of the seedlings are again settling down to their normal aerobic courses.

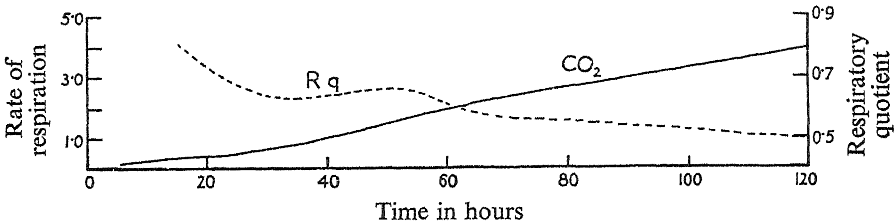

FIG. 8—*Ricinus*, experiment 107

DISCUSSION

From an examination of the data set out above it is evident that the respiratory behaviour of the seedlings, under the experimental conditions used, is similar in a general way for all the three species used. The change in atmosphere from air to nitrogen always results in a rapid fall in the rate of carbon dioxide output. The extent of this fall shows no constant value, it differs widely between individual seedlings of the same species. Respiration in nitrogen of all seedlings appears to follow a falling course, the rate of fall may be slow as with *Cucurbita* and with the variety of *Helianthus* used in experiments A 15 to A 22. On the other hand, as is shown by *Ricinus*, the fall may be slow for seedlings which are at an early stage of development and which are not respiring very actively, and rapid where seedlings are older and metabolically more active. This same rapid fall is also exhibited in the behaviour of the more active variety of *Helianthus* used in experiments 172, 193, 194, 195, and 196. Transference of the experimental seedlings from nitrogen to air always results in an initial rapid rise in the carbon dioxide output usually followed by a more gradual rise as the normal respiration rate, appropriate to the developmental condition of the experimental seedling, is reached.

A further brief examination of the respiratory behaviour of the seedlings in nitrogen may now be made. The initial rapid fall which accompanies the change from air to nitrogen is what we should expect to happen in

a change from a simple oxidation of glucose to carbon dioxide and water, to an anærobic oxidation of it to carbon dioxide and alcohol. From the fact that the behaviour, of all three of the seedlings used, is similar in respect to this initial fall we might reasonably conclude that the actual respiratory substrate is at least similar for each. That the substrate is not fat is evident because the available supply of it appears to become exhausted after prolonged maintenance of the seedlings in absence of oxygen. This gradual exhaustion of substrate is deduced from the falling course of the respiration in nitrogen. It would seem that this falling course is not due to the accumulation of toxic substances in the respiring cells for then one would not expect the seedlings to resume so rapidly, as they do, their normal respiratory activity when they are again given an adequate supply of oxygen.

We know that absorption of oxygen must accompany the conversion of fat to sugar, consequently it would therefore appear that part, at least, of the oxygen absorbed in the cases of *Helianthus*, *Cucurbita*, and *Ricinus* is used for the conversion of fat to sugar. Further it would appear that fat, as such, does not directly form the respiratory substrate. Whether oxygen is or is not necessary for the maintenance of some additional pre-glycolytic stage cannot be inferred from these experiments, but in this connection it is of interest to note that Blackman (1928) concluded that it was for the respiration of apples. One respect, however, in which the respiration of developing seedlings differs markedly from that of apples is that in the former change from aerobic to anærobic conditions always results in a reduction in the rate of carbon dioxide production. In senescent apples, Blackman and Parija (1928) found that a change from aerobic to anærobic conditions brought about an increase in carbon dioxide output. Hill (1913) found that ripe cherries, blackberries, and grapes respired as actively anærobically as aerobically, but, on the other hand, he found that growing tissues such as green peaches and germinating wheat respired more than twice as vigorously in air as they did in nitrogen and in hydrogen. It would therefore seem possible that the respiratory processes of senescent fruits differ in some important respect from those of actively growing tissues. The explanation of this difference will probably only be forthcoming when chemical investigations into the problem have been carried out.

With regard to the respiratory quotient, the course followed by this, immediately after the change from anærobic to aerobic conditions, is of considerable significance. Again, in respect of the respiratory quotient, all the three species used in these investigations show a similarity of behaviour on general lines one to another, although there are certain

differences in detail. With young seedlings of *Helianthus*, *Cucurbita*, and *Ricinus*, shortly after the transference of the seedlings from air to nitrogen is made, the value of the quotient falls to a comparatively low figure from which it may rise for a time reaching a maximum, after which it again falls, apparently to take up the normal developmental course which characterizes the respiratory behaviour of the seedlings.

With older seedlings of *Helianthus* as described on p. 158 this rise to a maximum with a subsequent fall is absent, the rise instead being continued. It may be that this continued rise is also merely a resumption of the developmental course which does follow an upward trend as the reserves of fat in the cotyledons become exhausted.

During the anærobic experimental period the respiratory quotient is infinity as no oxygen is being absorbed. As the gas surrounding the seedlings is changed from nitrogen to air the respiring cells begin to absorb oxygen and consequently the respiratory quotient falls from an infinitely high value to the initial low value mentioned above. This absorption of oxygen by the cells must be very rapid; in fact, in *Cucurbita* it is so rapid that it is frequently unrecorded by the measuring apparatus. It is hence suggested by these respiratory quotient data that during anærobiosis some product which has a very strong affinity for oxygen is formed which, on admission of oxygen to the respiring cells, becomes rapidly oxidized. This initial oxidation results in the rapid rise in carbon dioxide output which marks the change from anærobic to aerobic conditions. The subsequent rise in the respiratory quotient to a maximum might be explained by the resumption of equilibrium in the pre-glycolytic stage of the process which had been stopped during the experimental period in absence of oxygen. This final rise in the respiratory quotient to a maximum was obtained by Flieg (1922), who worked with cultures of *Aspergillus niger* on triolein and on castor oil, respiring respectively in air, nitrogen, and air. He does not, however, give any data in respect to the rate of the initial fall to a minimum value mentioned above.

Experimental data, similar in nature to those that have been dealt with in this paper in connection with the germination of seeds which store fat, have been obtained for a number of germinating seeds with carbohydrate food reserves. These will be described and discussed in the next number of this series of researches.

Summary

Measurements have been made of the changes in respiration intensities and in the values of the respiratory quotients that take place when seedlings

of *Helianthus annuus*, *Cucurbita pepo*, and *Ricinus communis* are successively subjected to atmospheres of air, nitrogen, and air. For *Helianthus* and *Ricinus*, respiration rate determinations were carried-out both with the katharometer and by the Pettenkofer method in order to examine the relative advantages and disadvantages of the two experimental methods.

A change in the surrounding gas from air to nitrogen, for each of the species examined, resulted in a rapid fall in the rate of carbon dioxide output which was followed by a less rapid fall which continued throughout the period of anærobiosis. The subsequent change from anærobic to aerobic conditions was accompanied by a correspondingly rapid rise in the respiration rate to the normal " air-line " value appropriate to each particular seedling.

The values of the respiratory quotients, following on the changing of the seedlings from respiration in nitrogen to respiration in air, first of all fell rapidly to a relatively low figure from which they rose and finally assumed their normal aerobic developmental courses.

Possible tentative explanations of the facts observed are discussed.

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612.621.5 : 599.33

The Reaction of Anœstrous Hedgehogs to Experimental Conditions

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(*Communicated by Sir Henry Dale, Sec. R.S.—Received June* 7, 1934)

INTRODUCTION

Male and female hedgehogs both have a well-defined anœstrus under natural conditions (Allanson, 1934, and Deanesly, 1934), and it seemed possible that this species, like the ferret, would retain the period of quiescence in the laboratory, and would thus be useful for work with gonad-stimulating preparations.

Herlant (1932), who gives a full bibliography, attempted to stimulate male hedgehogs during anœstrus experimentally, with a view to proving the dependence of the secondary sexual characters on the interstitial cells of the testis and not on the germinal epithelium. He injected normal, unilaterally castrated and irradiated hedgehogs during the winter with urine of pregnancy, and obtained slight growth and histological changes in the accessory organs. The interstitial cells showed increased development, but the changes in the germinal epithelium were negligible. The extent of the reaction could be correlated roughly with the amount of urine of pregnancy injected. In this connection it should be noted that the injections described in the present paper corresponded to far larger quantities of urine of pregnancy than were used by Herlant.

Attempts to breed hedgehogs in captivity during 1930–32 were unsuccessful (*cf.* Hubrecht, 1889), and it was therefore somewhat surprising to find from analysis of the records of untreated animals that some of those kept in captivity in winter for more than a few days were appreciably better developed than those killed straight from the field. Such animals were few in number, and were discarded from the normal series recently described, but they gave an early indication that any experimental work on anœstrous hedgehogs would have to be very carefully controlled by the examination of untreated animals kept in captivity for an equal length of time.

There was no real hibernation in the hedgehogs kept in the laboratory, although some animals slept for long periods at a time. Food provided daily was eaten regularly. In hedgehogs in the field, the extent of hibernation is known to vary, probably according to weather and locality (Barrett-Hamilton, 1911; Mathias, 1929) but the animals certainly remain inactive and comatose for long periods between December and March.

The present paper records the results obtained on male and female hedgehogs subjected during the winter anœstrus to (*a*) ordinary laboratory conditions, (*b*) an environmental temperature of 70°–75° F, (*c*) evening illumination, (*d*) administration of œstrin, (*e*) administration of urine of pregnancy extracts.

II Methods and Material

Hedgehogs were obtained from the field, and immediately assigned to one or other of the experiments. Since the animals differed according to sex, age, body weight and time of coming into the laboratory, it was not always possible to arrange closely similar pairs of control and experimental animals, but the treated and untreated groups were made as alike as possible for each experiment.

All hedgehogs received the same diet of milk and horseflesh. The temperature under ordinary laboratory conditions was about 65° F.

The evening illumination was arranged as previously described for ferrets (Hill and Parkes, 1933).

Both ketohydroxy- and trihydroxy-œstrin were used. No trouble was experienced in making the injections ; the skin was raised with a pair of toothed forceps, and a long needle thrust down among the spines, through the skin and into the subcutaneous tissue.

Two different extracts of urine of pregnancy were injected : the first, U.P. 1 (given to the females HOH 6, 7, and 10), was part of the preparation described by Hill, Parkes, and White (1934), having a rabbit ovulation unit of 5 mg. The second extract, U.P. 8, given to the females HOH 14 and 15, and to the males, had a rabbit ovulation unit of 0·5 mg. It was supplied by courtesy of British Drug Houses, Ltd., as was the œstrin.

Dissection, weighing of the organs, and histological technique were carried out as usual, except that for some animals only one in six of the serial sections through the ovary were mounted. The weights of the organs of normal animals, plotted in figs. 1–4, are taken from the collection of hedgehogs already described.

III Anœstrus and the Transition to the Breeding Season in Normal Hedgehogs

(*a*) *Females*—Before describing the effect of various treatments on the anœstrous female hedgehog, it is necessary to give some account of the condition of the reproductive organs during the autumn and winter months when no ovulation occurs. The decrease in the size of the reproductive tract after the breeding season has been described in detail in an earlier paper, Deanesly (1934), and also by Courrier (1924).

The ovaries of parous hedgehogs show a slight shrinkage at the end of the breeding season, associated with the degeneration of the old corpora lutea. Follicular activity does not entirely cease in anœstrus, but between November and February the largest follicles are generally not more than 0·8 mm in diameter. The same applies to the much smaller ovaries of first-year non-parous animals. In parous hedgehogs the anœstrous uterus varies in size according to the degree of involution since the last pregnancy ; the range of weight in the winter months (0·34–0·83 gm) is shown in fig. 1 and Table I. The increase in weight at the next breeding season is not obvious until May. Between the beginning of November and the end of April the average uterus weight of 18 parous hedgehogs is 0·55 gm, or about half the breeding season size. In the typical anœstrous uterus the mucosa is shrunken and dense, and the glands are small. The vagina of the parous hedgehog appears inactive in anœstrus ; it is collapsed and shrunken, the glands are quiescent, and the epithelium irregular and generally thin. Owing to the difficulty of making a clean dissection, this organ was not weighed.

The best available index of the condition of the reproductive organs of female hedgehogs is the weight of the uterus, if the animals are first classified into parous and non-parous groups (figs. 1 and 2). In the latter the uteri weigh only 0·1–0·2 gm until the end of March, fig. 2, and the vaginæ are correspondingly small and undeveloped. The breeding season changes are similar in the two groups of animals, but begin earlier in the first-year hedgehogs, whose uteri increase rapidly after the end of March. The enlargement is brought about partly by œdema, but mainly by generalized growth of the tissues, especially the mucosa.

In the vagina there are also changes as the breeding season approaches; the epithelium becomes thickened and cornified, and growth and sloughing of its outer layers occurs in April, about a month before the first ovulation. Coincidently, the vaginal glands become active, and their

secretion is poured into the lumen. During this period of vaginal activity numerous follicles in the ovary reach œstrous size (diameter about 1 mm) and then disintegrate, but there is no appreciable change in the size of the ovary, which is somewhat variable.

(*b*) *Males*—The condition of the winter testis and accessory sexual organs of the male hedgehog have been described by Marshall (1911), Courrier (1927), and Allanson (1934). Production of spermatozoa ceases during September, and from October until the end of December the seminiferous tubules contain no stages later than primary spermatocytes. From January to March there is active preparation for the next breeding season, spermatocytes, and, towards the end of the period, spermatids, being produced in large numbers. Although a few spermatozoa may occasionally be found in the testis as early as January, they are not numerous until April, when full activity is reached by most adults. From October to February the testes weigh 0·9–2·5 gm ; in the breeding season they are usually more than 2·5 gm and many weigh over 5 gm, fig. 3. Testis weight, however, is only a rough index of spermatogenic activity, since testes weighing only 2 gm may be fully spermatic in the breeding season. In animals born in the previous year, the weight of the testes remains below that of the adults throughout the winter and spring, and full activity is attained later than in the adults.

The seminiferous tubules in the quiescent adult testis measure 130–150 μ in diameter ; in full activity they measure 190–210 μ. The interstitial cells also undergo seasonal size variation ; in anœstrus the area is about 100 sq. μ, in the breeding season over 200 sq. μ.

The weights of the winter and breeding season epididymides show a difference of about 100%. In the adult they weigh 0·3–0·5 gm from October to late March, and 0·6–0·9 gm during the breeding season. The average diameter of the tube is 120–140 μ during quiescence, 180–220 μ from May to August. Spermatozoa do not appear in the epididymis until the middle of April.

In the accessory sexual glands there is a marked difference between the anœstrous and breeding season appearance, due to rapid growth and involution. The seasonal variation in the weight of the seminal vesicles is shown in fig. 4 ; similar changes occur in the other glands. The seminal vesicles from October to March weigh 2–4 gm, but increase during April to as much as 30 gm when full of secretion. Prostate glands weigh 1–2 gm in anœstrus, and up to 9 gm from May onwards. Cowper's glands vary from 1 gm or less in anœstrus, to 10 gm at their maximum. Most of the remarkable increase in size takes place after the testis is fully active, so that animals are found at the beginning of the

breeding season in complete spermatogenesis, but with accessory glands differing little from those in anœstrus.

IV The Reaction of Female Hedgehogs to Experimental Conditions

(*a*) *Captivity*—Twelve parous and nine non-parous hedgehogs were kept in captivity for various periods between October and the end of March. These animals are listed in Table I. Owing to the variation in body weight, season and length of captivity, the material is by no means

Table I—Female hedgehogs kept in the laboratory from November to March. Those lettered HL are the controls of the animals receiving evening illumination, and those lettered HOH the controls of the injected hedgehogs ; the others are animals kept in captivity in 1930 and 1931.

No. of animal	Body weight gm	Killed	Days in laboratory	Uterus weight gm	Ovaries
HL 24	710	December 18	30	1·05	+++
HL 19	700	December 22	43	1·32	C.L. +++
HH 18	1050	January 16	44	0·90	++
HOH 13	580	February 10	17	0·85	+
HH 24	600	February 14	12	0·63	+
HL 7	630	February 21	40	0·56	++
HL 11	700	March 7	49	0·88	+
HL 5	700	March 9	56	0·94	+++
HL 8	440	March 17	64	0·53	++
HL 6	680	March 17	63	0·96	+++
HH 26	700	March 18	60	0·62	+
HL 18*	530	December 8	29	0·10	+++
HH 16*	300	December 11	8	0·20	+
HH 8*	650	December 18	120	0·37	+
HL 23*	590	December 22	34	0·58	+++
HL 21*	500	December 27	43	0·14	++
HOH 9*	300	January 21	5	0·08	+
HOH 3*	410	January 30	14	0·10	+
HOH 4*	540	January 30	14	0·26	+
HOH 5*	600	January 30	14	0·65	+++

Tables I–III abbreviations

\+ follicles under 0·9 mm diameter.
++ follicles 0·9 – 1 mm diameter.
+++ follicles over 1 mm in diameter (œstrous size).
R.F. ruptured follicle. C.L. corpora lutea.
U.P. 1 and U.P.8—urine of pregnancy extracts.
* immature or non-parous.

homogeneous, but certain general conclusions may be drawn. Most of the hedgehogs increased in weight while in the laboratory, and some of them were very fat when killed. The condition of the reproductive organs at death ranged from that of normal anœstrus to that of the breeding season, but the majority of both parous and non-parous hedgehogs were approaching the latter. In some animals twelve or fourteen days' captivity had produced distinct changes in the reproductive tract. The ovaries of the active animals contained large follicles, some normal and others degenerating; the uteri were well developed and uterine glands showed mitoses, and the vaginæ had enlarged and contained secretion and cornified debris. There was a general resemblance to the hedgehogs taken from the field in April and May which had not yet ovulated. The uterus weights are plotted in figs. 1 and 2; most of the uteri are heavier than those of the normal series belonging to corresponding months, and some have actually attained full breeding season size.

Of the non-parous hedgehogs, three were hardly different from the field material, although one had been as much as 43 days in the laboratory. The others showed various stages of uterine enlargement, up to that reached in May by normal first-year animals, which have just begun to ovulate. The non-parous hedgehogs, in which the reproductive tract developed most in captivity, were the largest of the series, but increase in body weight alone does not produce this in normal animals in anœstrus; a hedgehog weighing 520 gm was taken from the field in January, with an undeveloped reproductive tract (uterus weight 0·09 gm). None of the untreated, non-parous hedgehogs ovulated during anœstrus in captivity.

In 8 out of 12 of the untreated parous hedgehogs killed between November and March, ovaries, uterus, and vagina showed clear indications of breeding season activity. Almost all these hedgehogs had large follicles and several appeared to be œstrous, but only one animal, HL 19, killed on December 22, had actually ovulated, the left ovary containing a recently formed corpus luteum and three large follicles. By contrast, other parous hedgehogs (HL 7 and 8 and HH 26) remained quiescent, although they were kept long in the laboratory.

(*b*) *Light*—Eight parous and three non-parous hedgehogs were given six to seven hours' evening illumination daily, during anœstrus, for periods varying from 19–63 days (Table II). In most of these animals there were the changes in the reproductive organs already described. The weights of the uteri are shown in figs. 1 and 2; they tend to be rather larger than those of the control groups, the parous uteri averaging 1·13 gm against 0·84 gm in the unlighted controls. The vaginæ also showed breeding season changes, and most of them, like the uteri, had the characteristic

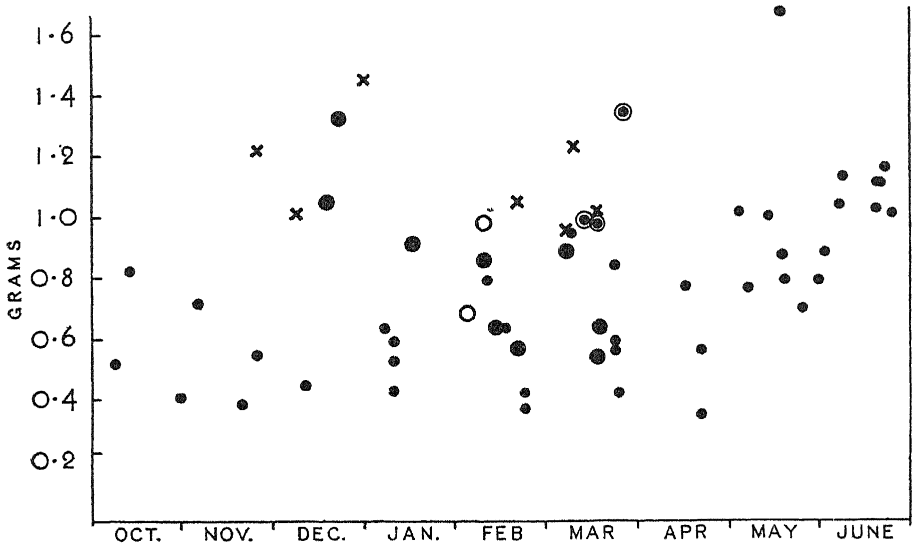

FIG. 1—Weights of uteri of parous hedgehogs between October and June.
• Normal (field) ; ● captivity ; × light ; ○ œstrin ; ◉ U.P.

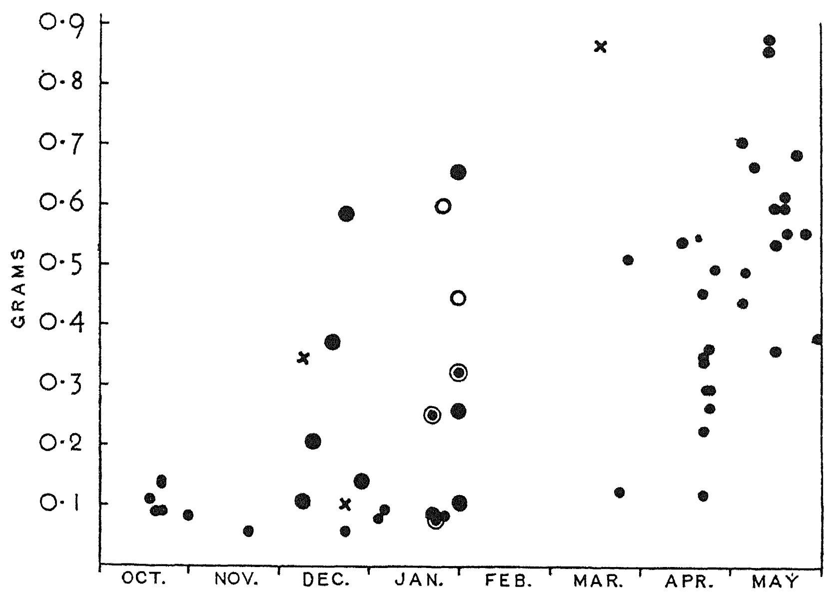

FIG. 2—Weights of uteri of non-parous hedgehogs between October and May.
• Normal (field) ; ● captivity ; × light ; ○ œstrin ; ◉ U.P.

Table II—Female hedgehogs receiving additional light

No. of animal	Body weight gm	Killed	Days treated	Uterus weight gm	Ovaries
HL 14	720	November 25	31	1·22	+++
HL 15	660	December 8	44	1·01	+++
HL 20	1100	December 30	44	1·45	++
HL 3	690	February 21	40	1·04	+++
HL 12	900	March 7	49	0·95	+++
HL 1	1100	March 9	56	1·22	C.L. +++
HL 10	890	March 17	63	1·01	+++
HL 17*	450	December 8	29	0·34	+++
HL 22*	380	December 22	29	0·10	+++
HL 13*	750	March 17	59	0·86	C.L. +++

œstrous appearance. Large follicles, some of them degenerating, were numerous in the ovaries, but only two animals had ovulated—a parous hedgehog killed on March 9 after 56 days' treatment, and a non-parous animal killed on March 17 after 59 days' treatment. Although these ovulations are comparatively late in anœstrus, they are, nevertheless, about a month before the normal female breeding season. In view of the ovulation in the unlighted control hedgehog described above, however, they cannot be considered as evidence of stimulation by additional light. HL 1 contained three corpora lutea and HL 13 only one corpus luteum in the two ovaries, while the average number at ovulation is five in the normal breeding season.

(*c*) *Injection of œstrin*—Two parous and two non-parous hedgehogs were injected daily with 100γ of œstrin in 4% alcohol, the former receiving ketohydroxy-œstrin and the latter trihydroxy-œstrin ; the details and the uterus weights are in Table III, and figs. 1 and 2. In the parous

Table III—Female hedgehogs injected with œstrin and urine of pregnancy extracts

No. of animal	Body weight gm	Daily injection	No. of days	Killed	Uterus weight gm	Ovaries
HOH 12	600	100γ œstrin ..	8	February 5 ..	0·68	+
HOH 11	600	,, ..	13	February 10 ..	0·97	+
HOH 1*	420	,, ..	9	January 25	0·60	+
HOH 8*	400	,, ..	14	January 30	0·44	+
HOH 6*	300	50 mg, U.P. 1	5	January 21	0·25	C.L., etc.
HOH 7*	300	,, ,,	6	Died January 22	0·08	R.F.
HOH 10*	280	,, ,,	14	January 30	0·32	C.L., etc.
HOH 14	560	2 mg, U.P. 8	4	March 14	0·98	C.L., etc.
HOH 15	660	,, ,,	15	March 26	1·33	C.L., etc.

hedgehogs, which received the less active trihydroxy-œstrin, the uterus and vagina were not very different from those of the control HOH 13 (Table I), which also showed pre-breeding season changes. None of the œstrin-injected hedgehogs had full enlargement of the vagina, but only partial growth and cornification such as normally occurs before the breeding season has begun. The most conspicuous changes were in the non-parous hedgehog HOH 1, where the uterus reached breeding season size and appearance after nine days' injection. HOH 5 among the controls had a very similar uterus, but this was a much larger animal, and had been five days longer in captivity. The other non-parous controls had small uteri. The ovaries, as would be expected, showed no reaction to the injections ; follicles were normal.

(*d*) *Injection of urine of pregnancy extract*—Two parous and three non-parous immature hedgehogs, Table III, were given daily injections of five rabbit ovulation units. All three non-parous animals ovulated. Two were killed after 5 and 14 injections, and the other died after 6 injections. HOH 7, which died, had three recently ruptured follicles in the ovaries ; the other two animals had more than one set of corpora lutea. In HOH 6 the corpora lutea approximated to those found early in the breeding season in the normal unmated hedgehog ; the lutein cells were small, and there was often a central blood clot. The theca, however, which disappears as such from the normal corpus luteum once the vascularization is complete, had persisted and become partially luteinized, a condition found in ovaries of other animals injected with urine of pregnancy extract (Hill and Parkes, 1930). The majority of the corpora lutea in HOH 6 had been formed after ovulation, but some were atretic and contained the remains of ova. A few small follicles were also luteinized. A large cystic follicle and smaller, pre-ovulation follicles were also present in the ovaries.

Uterus and vagina were still rather undeveloped, but the former showed growth and œdema, and the epithelium of the latter had thickened and begun to slough.

In HOH 10, which was injected for 14 days, the corpora lutea were larger and more numerous than in HOH 6 and 7. The lutein cells developed from the granulosa resembled those found in normal corpora lutea of pseudo-pregnancy, and were distinct from the luteinized cells of the theca, which were also present. The ovary also contained atretic corpora lutea. The increase in weight of the ovaries compared with the controls was not very marked—about 20%. The largest non-parous uterus, that of HOH 10, was only half up to breeding season size, fig. 2.

The two parous hedgehogs, receiving five rabbit ovulation units daily of the more concentrated extract U.P. 8, both showed an increase in the weight of ovaries and uterus, fig. 1. HOH 14 had only four injections, but histologically its ovaries resembled those of HOH 10, with ruptured follicles and developing corpora lutea. HOH 15, after 10 injections, had very large ovaries, 0·24 gm, nearly up to the maximum breeding season size, but the corpora lutea produced by repeated ovulations were numerous rather than large, and luteinization of the theca interna was again conspicuous. The Fallopian tube contained ova. The mammary gland showed no reaction to the injections.

V The Reaction of Male Hedgehogs to Experimental Conditions

The data for male hedgehogs subjected to the various experimental conditions are given in Table IV. In figs. 3 and 4 the weights of testes and seminal vesicles of the adult animals are compared with those of adults from the field.

(*a*) *Captivity*—Nine male hedgehogs were kept in captivity for periods varying from 17 days to 8 months. Seven of the animals were definitely immature, while two, HTH 21 and HH 246, from body weight and the appearance of the accessory sexual organs, were probably more than a year old. In HTH 21, killed on December 27 after 42 days in the laboratory, the testes were fully active, and spermatozoa were in the epididymis. The weights of testes and epididymides were of breeding season standard, but the small diameter of the seminiferous tubules and epididymis tube indicated that activity was only recent. The interstitial cells were large and glandular, but the accessory sexual glands were still anœstrous in size. HH 246, killed on February 29, had been for two months in an outdoor compound ; the reproductive organs were in all respects less advanced than in HTH 21, but there was full spermatogenesis and spermatozoa in the epididymis. Five of the immature males were kept in the laboratory for 17 days, and killed on January 27. The weights of all the reproductive organs were higher than for immature animals from the field, and the testes in HTH 3, 4, and 5 were fully spermatic. Spermatozoa were found in the epididymis, but the testis tubule diameter and the state of the germinal epithelium indicated that they had only recently developed. In HTH 6 spermatogenesis was less advanced, and HTH 7 differed little from the normal.

Two animals, HH 5 and HH 4, arrived in the laboratory with their mother in July, and were killed in the following February and March.

Table IV—Male hedgehogs receiving experimental treatment

No. of animal	Body weight	Treatment	Days treated	Killed	Testis: Weight (2)	Testis: Mean tubule diameter	Testis: Stage of spermatogenesis	Testis: Mean area of interstitial cells	Epididymis: Weight (2)	Epididymis: Mean tube diameter	Weight of seminal vesicles	Weight of prostate glands	Weight of Cowper's glands
	gm				gm	μ		sq. μ	gm	μ	gm	gm	gm
HTH 21	600	Normal laboratory conditions	42	Dec. 27 ..	4·75	171	SPZ	258	0·78	166	5·60	1·18	2·35
HTH 3*	480	,,	17	Jan. 27 ..	2·92	145	SPZ	221	0·41	141	2·10	0·51	0·77
HTH 4*	390	,,	17	Jan. 27 ..	2·31	148	SPZ	238	0·34	118	0·76	0·20	0·42
HTH 5*	440	,,	17	Jan. 27 ..	2·80	147	SPZ	197	0·34	126	0·21	0·06	0·19
HTH 6*	400	,,	17	Jan. 27 ..	1·80	150	SPT	166	0·29	—	0·44	0·14	0·28
HTH 7*	410	,,	17	Jan. 27 ..	1·41	122	SPT	193	0·20	103	0·15	0·06	0·09
HH 5*	420	,,	210	Feb. 13 ..	3·32	164	SPZ	—	0·61	—	4·13	0·95	—
HH 246	740	,,	60	Feb. 29 ..	3·45	178	SPZ	211	0·71	162	3·63	1·01	2·18
HH 4*	330	,,	240	Mar. 11 ..	3·17	163	SPZ	—	0·55	160	2·90	0·90	—
HTH 16	1160	Heat	28	Nov. 24 ..	3·05	149	SPT	166	0·62	149	2·15	0·96	1·04
HTH 17	730	,, ...	42	Dec. 9	2·70	171	SPZ	364	0·75	166	17·37	3·02	5·20
HTH 19	680	,, ...	39	Dec. 20 ..	1·40	126	SPC	74	0·38	117	1·17	—	0·65
HTH 13	910	Light	28	Nov. 24 ..	5·12	195	SPZ	269	0·81	154	9·40	1·60	2·55
HTH 20	570	,, ...	28	Dec. 9 ..	2·45	180	SPZ	320	0·43	171	2·25	0·45	0·90
HTH 12	740	,, ...	42	Dec. 9 ..	3·05	169	SPZ	279	0·68	162	12·93	1·10	3·22
HTH 14	620	5 mg daily ..	28	Nov. 24 ..	2·28	205	SPZ	307	0·45	171	0·69	0·26	0·56
HTH 15	840	U.P. 8	28	Nov. 24 ..	3·70	200	SPZ	310	0·83	183	28·55	7·55	10·39
HTH 23	650	10 mg daily.. U.P. 8	12	Dec. 9	3·82	171	SPZ	288	0·68	149	6·06	1·52	1·82

Abbreviations.—* Immature. U.P. 8, Urine of pregnancy extract. SPC, Spermatocytes. SPT, Spermatids. SPZ, Spermatozoa.

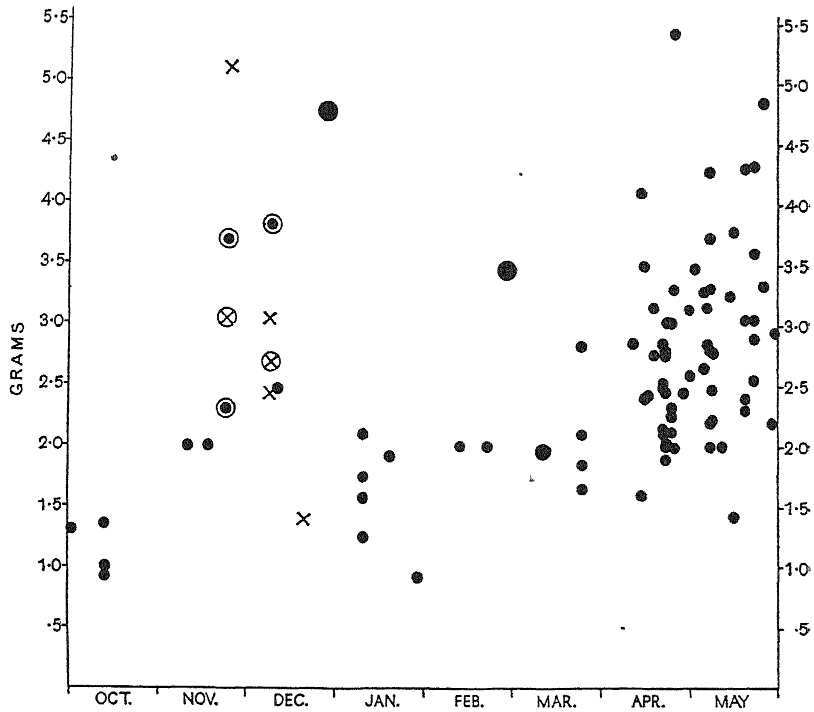

FIG. 3—Weights of testes of adult hedgehogs between October and May.
• Normal (field) ; ● captivity ; X light ; ⊗ heat ; ◉ U.P.

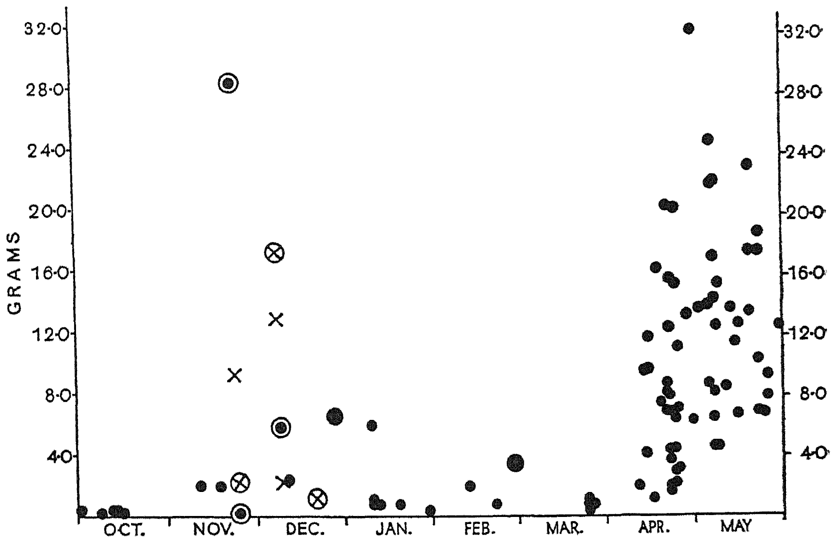

FIG. 4—Weights of seminal vesicles of adult hedgehogs between October and May.
• Normal (field); ● captivity ; X light ; ⊗ heat ; ◉ U.P.

The testes were fully spermatic and the epididymides crowded with spermatozoa, but the accessory organs, although large for immature animals in the same months, were little developed.

(*b*) *Warmth*—Three adult males were kept for periods of 28–42 days in an environmental temperature about 10° above the normal laboratory one. In HTH 16, killed on November 24 after 28 days' treatment, the testes and epididymides were heavy, but the accessory glands were similar in weight and appearance to those of the anœstrous field animal. Histologically, the testes showed the beginnings of activity, half of the tubules in a transverse section containing small numbers of spermatids which were occasionally elongated. The epididymis was well developed, but contained no spermatozoa. The interstitial cells were larger than in the normal November testis. HTH 17 was killed on December 9 after 42 days' treatment. The testes and accessory organs were of full breeding season size, spermatogenesis was proceeding actively, and the epididymis was crowded with spermatozoa. In HTH 19, killed on December 20 after 39 days' treatment, however, the reproductive organs showed no advance over those of December animals in the normal series ; the testis tubules held no stages later than primary spermatocytes, and the accessory organs were anœstrous.

(*c*) *Light*—Of the three adult males given six to seven hours' evening illumination daily, one (HTH 13) was killed on November 24 after 28 days' treatment, and two (HTH 20 and 12) on December 9 after 28 days' and 42 days' treatment respectively. The testes of all three animals were in full spermatogenesis, the seminiferous tubules and interstitial cells were of breeding season size, and spermatozoa were present in the epididymis. In HTH 13 and 12 the accessory glands had been stimulated to about one-fourth of full breeding season development, but in HTH 20 they had remained quiescent.

(*d*) *Injection of urine of pregnancy extract*—Two animals (HTH 14 and 15) were given daily injections of 5 mg of U.P. 8 for 28 days and killed on November 24. In both the testes showed full spermatogenesis, and the epididymides contained spermatozoa. The weights of testes and epididymides were typical of the breeding season, but the accessory organs differed in the two animals. In HTH 14 they had remained unaffected, but in HTH 15 full breeding season size had been reached, the weights approximating to the highest in the normal series. In HTH 23, killed on December 9, full spermatogenesis had been produced after 12 days' injections of twice the amount given to the other two, and spermatozoa had reached the caput epididymis. The accessory glands showed a slight but definite increase over the normal.

VI Discussion

(*a*) *Response of female hedgehogs to experimental conditions*—The characteristic anœstrus lasting from November till April in female hedgehogs was readily modified in animals kept in captivity, presumably by environmental influences such as food and warmth. The changes in the reproductive organs, however, were generally confined to the maximum development of the follicular phase of the normal cycle ; the fully active condition in which spontaneous ovulation takes place was rarely attained. Hedgehogs receiving evening illumination for six to seven hours daily failed to show any greater activity than the control hedgehogs kept in the laboratory (contrast Bissonnette, 1932). An untreated animal ovulated in December, and two receiving extra light ovulated in March. Other hedgehogs showed full œstrous development of the uterus and vagina, but failed to ovulate, while some remained anœstrous under similar conditions. The injection of œstrin and urine of pregnancy extract produces changes comparable to those described in other mammals ; the results in general confirm those of earlier workers (Courrier, 1924, Herlant, 1931). Herlant, however, reports failure to induce ovulation in an immature hedgehog with urine of pregnancy in January, and abundance of atretic follicles in two injected parous hedgehogs in October.

(*b*) *Response of male hedgehogs to experimental conditions*—Complete spermatogenesis and glandular development of the interstitial cells and epididymis was readily induced in the anœstrous hedgehog, but the reaction of the accessory glands varied greatly in some cases. Ordinary laboratory conditions caused no stimulation, but longer captivity would probably have produced full growth of the glands in the adults, since there is normally a period of two to three weeks at the beginning of the breeding season when the testes are active but the accessory glands little enlarged. Light and warmth and injections of urine of pregnancy extract caused rapid growth of the accessory glands, and a definite reaction to urine of pregnancy was produced after 12 daily injections.

Individual variation probably accounts for the difference in the response of the accessory organs of HTH 14 and 15 receiving the same amount of urine of pregnancy extract, and in HTH 19 and 17 after nearly identical periods of extra warmth, but those with the smaller accessory organs may have been immature.

Although the numbers of animals in each type of experiment were small, the results show clearly that the reproductive organs of the anœstrous hedgehog are easily stimulated to breeding season development and

activity. The hedgehog thus differs from the ferret, in which full spermatogenesis cannot be induced by extra food and warmth, and in which pregnancy urine extracts have as yet been ineffective on the testis. To bring about full activity in the ferret nearly three months' evening light is required (Allanson, Rowlands, and Parkes, 1934). The difference between the two animals is not altogether surprising, since the testis regresses less in anœstrus in the hedgehog, where it is abdominal, than in the ferret ; the seminiferous tubules show a smaller size decrease and retain several rows of spermatocytes throughout the winter.

The experiments described above were designed and carried out in collaboration with Dr. A. S. Parkes, F.R.S.

VII Summary

Between October and March anœstrous male and female hedgehogs were kept in the laboratory (*a*) without treatment, (*b*) at an environmental temperature of 70°–75° F, (*c*) with additional evening illumination, (*d*) receiving injections of œstrin, (*e*) receiving injections of urine of pregnancy extract.

Changes characteristic of the breeding season were induced in the majority of experimental animals.

The females showed follicular growth and enlargement of the uterus and vagina, such as normally does not occur until April or May. In several animals there was full œstrous development, but only three ovulated.

In untreated males kept in the laboratory, the testes became active in December. Full activity was reached 2 4 weeks earlier by the animals given extra warmth, extra light or injections of urine of pregnancy extract.

The accessory glands were like the normal anœstrous ones in the untreated males, but were stimulated by the experimental conditions.

The anœstrus of the hedgehog is therefore unstable in comparison with the definite period of quiescence of animals like the ferret. This conclusion is of some theoretical importance in its bearing on the fixity or non-fixity of the breeding season in various species, especially in those with a wide climatic range.

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Discussion on Methods of Measuring and Factors Determining the Speed of Chemical Reactions

Opening address by Professor A. V. Hill, F.R.S.

(June 14, 1934)

(Printed Abstract, circulated before the Meeting)

This discussion is planned on lines leading ultimately to an attack on biological problems ; the methods and results, however, are necessarily chemical and physical. To limit its scope, and to keep it within range of biological phenomena, reactions in an aqueous medium must be regarded as of chief interest.

The study of chemical velocity is important in biology for a special reason, namely, that the systems involved, though heterogeneous, are so small that diffusion may be relatively unimportant. In all diffusion equations the quantity involved is kt/l^2, where k is diffusion constant, t is time (sec), l is distance (cm). Taking k as typically of the order of 10^{-5}, l as 10^{-4} — 10^{-5} cm, t is of the order of 10^{-3} to 10^{-5} sec. Clearly reactions of any but the highest velocities inside single cells are not much hindered by diffusion unless impermeable surfaces intervene. In such small systems, moreover, surface phenomena and, as the late W. B. Hardy would have insisted, surface effects transmitted to a distance, are bound to be relatively more important.

The "time-scale" of vital phenomena is a matter of fundamental significance. The velocity of transmission of a nerve impulse may vary in the ratio of 10^4 to 1 from one animal to another ; the speed of muscular contraction in the same ratio in fibres structurally similar and of the same size. The latter variation at least must be a matter of physical or chemical difference of (as yet) quite unknown nature. The rate of resting metabolism varies greatly from one warm-blooded animal to another (particularly from small to large), as also does the rate of growth and the rate of senescence. A rise of temperature quickens vital processes as it does most chemical reactions ; but its effect is not the same on them all, consequently a constant temperature is necessary (as in warm-blooded animals) not only for a constant time-scale but for a constant relation between the several functions. Other agencies, *e.g.*, drugs or ions, affect the speed or frequency of vital reactions, perhaps, however, by altering the level at which periodic instability is attained in some chemical system continuously running in one direction.

The participation of enzymes, important as it is, is not the only factor. The loading and unloading of O_2 and CO_2, by the blood, in lungs and tissues involve the accurate adjustment of a number of reactions, only one of which is known to depend upon an enzyme. The great increase in the heat production of a resting muscle caused by keeping it stretched, and the fact that the amount of energy liberated by a contracting muscle (and therefore the velocity of the chemical reaction involved) may be greatly diminished when the muscle is allowed to shorten without load, but increased when mechanical work is done, may depend upon the properties either of oriented molecules in surfaces or of the organized system, whatever it is, whose existence is shown by double refraction, but scarcely upon those of enzymes alone.

It seems likely that all nervous transmission across synaptic boundaries (discontinuities between conducting cells) is associated with the rapid liberation, and the equally rapid destruction, of specific chemical substances. With acetyl-choline the process of destruction is diminished or prevented by a low concentration of eserine—by its specific effect on the esterase responsible for the breakdown. The retardation of reactions may provide a special chapter in the subject.

The electrical phenomena characteristic of all vital activity, and particularly of the transmission of messages, raise the question of the velocity of ionic reactions. Some of the electrical phenomena are undoubtedly equilibria—*e.g.*, the "injury" potential of non-medullated nerve varies with the logarithm of the K ion concentration. Others—the "action current," for example—clearly are not equilibria. Even if

ionic processes are extremely rapid—and are they necessarily so when enormous molecules are involved?—may they not be conditioned, as in the case of the bicarbonate ion, by non-ionic processes occurring at some stage in the reaction?

The great effect of light on the velocity of the $COHb \rightarrow CO + Hb$ reaction suggests that similar reactions in solution should be explored.

The speed of reaction may be altered if the molecules involved have recently taken part in another reaction. This might provide a mechanism for synthesis. For example, if the velocity of reaction of A with B recently formed were considerably different from that of A with B formed long before, the equilibrium of the reaction $A + B \rightleftarrows AB$ might be displaced by the use of recently formed B. Many of the substances involved in vital activity exist only for a short time in intermediate reactions: may these molecules during their short existence possess special properties and so pass in directions and with velocities otherwise not possible?

Living objects show the kind of molecular regularity which is revealed by X-ray analysis. The rate of formation of such regularities, whether in surfaces or in bulk, and the rate of transformation from one regular state to another (is this the basis of muscular contraction?) must depend upon molecular constitution in ways not yet known.

Remarks at the Discussion

I will take as read the printed abstract which is in your hands. My object in introducing this discussion is to indicate how important the conception of the velocity of chemical reactions is in various types of biological work, and to emphasize that the realization that the velocities of quite rapid chemical reactions can be measured has led already to important results. In the further development of this subject the biologist will need all the resources of chemistry and physics, and one of the chief objects, so far as I am concerned, is to try to invoke the help of chemists, physicists, and physical chemists in solving these difficult problems.

I have given various examples in the circulated paper. An example of practical interest is that of carbon monoxide poisoning. It has always been said that carbon monoxide combines with hæmoglobin, and brings about asphyxiation, by reason of the fact that it has a greater "affinity" than oxygen for hæmoglobin. That statement is far from the truth. The velocity of combination of carbon monoxide with hæmoglobin is actually less than the velocity of combination with oxygen. The reason

why the combination goes very much further is simply that the rate of breakdown of the compound of carbon monoxide with hæmoglobin is much less. In discussing the reactions of carbon monoxide, carbon dioxide, and oxygen with blood and tissues, these questions of velocities inevitably come in. We may go seriously astray if we regard the phenomena as depending only on equilibria. The case which I have instanced is one of many. Dr. Roughton could probably tell us of several other cases—for instance, of carbon dioxide where a consideration of equilibrium alone might lead us very far from the truth.

I made no mention of the effect of high pressures on the velocity of chemical reactions. Fortunately, we have here Dr. McKeen Cattell, from Cornell University Medical College, who has had experience of the effects of pressure on biological reactions, and I hope he may tell us some of the peculiar effects of high pressures (up to 1000 atmospheres) on the velocity of various chemical processes occurring in tissues.

I would elaborate one point mentioned in my paper, namely, the new and exciting phenomena which have recently been turning up in physiology, particularly in Sir Henry Dale's laboratory, concerned with the transmission of nerve impulses from one cell to another at what physiologists call synapses. This transmission has been found to depend upon the very rapid production and destruction of a chemical substance. The velocities concerned are necessarily very high ; in some ganglia the speed is such that the whole process must be over in a few hundred micro-seconds, and the concentrations are, I believe, of the order of 10^{-8} or less. When such fundamental matters as the transmission of nerve impulses across the gaps between separate conducting elements in the nervous system depend upon the production and destruction of chemical substances, one realizes the important part that a study of chemical velocities can play. One has a suspicion that the action of certain drugs on the nervous system may lie in the effect they have on the velocities of formation and destruction of the transmitting substance.

Another point mentioned in my note was the very peculiar effect discovered by Feng, that when a muscle is loaded, its rate of resting metabolism rises. Every living tissue shows a continual production of heat even in a state of complete rest. In the absence of oxygen it still shows a steady anærobic metabolism. The resting muscle, when loaded, may increase its resting metabolism several times, and that increase is greater, as von Euler has shown, if the muscle is alkaline, less if it is acid. It occurs the same whether oxygen is present or not, so that it is not an effect directly on rate of oxidation. There is a phenomenon which requires a physico-chemical—rather than a biological or functional—

explanation—a direct effect apparently of a mechanical stress on the rate at which a chemical reaction is proceeding. What the explanation may be one cannot say at present, but apparently the rate of breakdown of the combined creatin-phosphoric acid into creatin and phosphoric acid is modified by hanging a load on the system in which the reaction is occurring.

The problem we have to deal with is largely one of method. In the outer room we have had already demonstrations of some of the methods used. Each problem may require a new method. In biological investigation one of our chief difficulties is that direct chemical studies are almost certainly bound to injure the tissue in which the reactions are occurring. So far as possible, one wants to use methods, perhaps optical ones or thermal ones, recording phenomena which are known to be accompaniments of the chemical reactions, so as to study the reactions without interfering with the medium in which the reactions occur. For that reason optical methods in particular are valuable. One may instance recent experiments by Margaria on changes of hydrogen ion concentration in a contracting and relaxing muscle, followed with the aid of injected indicators, or experiments by von Muralt, in which the changes of double refraction occurring in muscular contraction are followed and recorded photographically by means of an interference method.

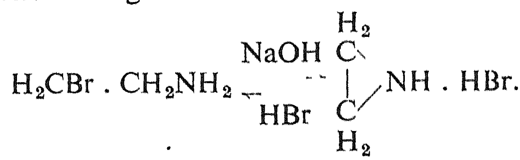
I refer in my note to the fact that the structure of living tissues is probably based upon a molecular organization which can be revealed by X-ray methods of analysis. If these methods could be very greatly quickened—for example, if they could be induced to follow the rapid changes that occur during muscular activity—the value of them to the physiologist would be greatly enhanced. At present I imagine it is not possible, without exposures of considerable duration, to get much evidence of molecular structure, but one may look forward to the time when a sort of cinematograph picture will be available of the molecular changes occurring during a single muscular contraction. The methods already available might be applied more fully to physiological problems, *e.g.*, to the investigation of the effect just mentioned, that resting metabolism may be increased by mechanical strain. A comparison of the X-ray structure of a muscle, with a small load upon it and with a large load, might give some indication of the reason why the velocity of the reactions going on is so much greater in the one case than in the other.

Many of these changes of structure are concerned with ionic processes. All living activity has electrical manifestations, and although one realizes that hydrochloric acid and soda react together with a velocity greater than one can measure, how far *all* ionic reactions are very rapid, and how

far in a system of very large molecules ionic reactions might be slower, we have still to be told. May we regard the comparative slowness of the electrical changes in living cells as being determined by the speed of the ionic reactions themselves?

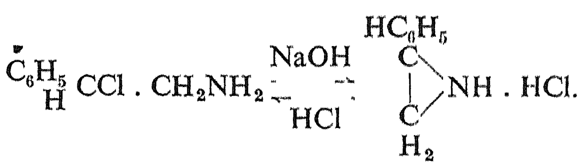
Although my own interest in this matter comes from the biological side, I realize that there is no limitation to it. If once one starts on a research one has to follow it up in whatever direction it may happen to lead. The problem is one of such variety that very different methods and very different ideas are required for tackling it.

Professor H. Freundlich: Chemical reactions are not only accelerated on surfaces—they may also be retarded. The following reaction, for instance, has been investigated* :—


(1)

The formation of the ring-compound is strongly retarded on charcoal, whereas the formation of the chain-compound is accelerated. This seemed to agree with what might have been anticipated from a thermodynamic point of view: the amine is adsorbed much more strongly than the salt of the ring-compound. A chemical equilibrium ought to be displaced on a surface in such a way that the formation of the more strongly adsorbable compound is favoured. So we might expect that the reaction of the amine would be retarded, its formation accelerated.

But the behaviour of a similar compound showed that this type of reaction is not explained so simply.† We investigated the reaction :—


(2)

The adsorbability of these aromatic substances is decidedly greater, though the difference between the adsorbability of the two substances is relatively smaller; nevertheless, the reaction of the amine is retarded

* Freundlich and Juliusburger, 'Z. phys. Chem.,' A, vol. **146**, p. 321 (1930).

† Freundlich and Salomon, 'Z. phys. Chem.,' A, vol. **166**, p. 179 (1933); 'Helv. Chim. Act.,' vol. **17**, p. 88 (1934).

much more strongly than reaction (1). Using the same amount of charcoal, the velocity of reaction (2) is decreased 10–50 times more strongly than in homogeneous solution ; in reaction (1) the rate of reaction is only 2–7 times smaller under the same conditions. The following data show how strong the retardation is in reaction (2) : in homogeneous solution about 50% of the amine is transformed in four minutes; in heterogeneous solution about 15% of the original amount of amine may be extracted from the charcoal after 18 hours.

We are obliged to enter more fully into the mechanism of these reactions. There are two limiting possibilities. One is that the reaction really goes on in the surface, that the surface behaves like any other medium in which the reaction takes place with a different velocity. In this case the surface of charcoal would be expected to behave like an organic liquid. One would expect such reactions, if the mobility of the adsorbed molecules is considerable. The other possibility is that the adsorbed molecules do not change at all ; the reaction may therefore be retarded fully on the surface, the only cause of the reaction going on being that a certain number of molecules are always in the solution, and the molecules there may react.

We have the impression that in the case of the brom-amine the reaction goes on in the adsorption layer, whereas in the case of the phenyl-compound the reaction of the molecules in the aqueous phase is mainly important. This seems to follow from the different behaviour of the temperature-coefficient. In homogeneous aqueous solution the influence of temperature is strong. The heat of activation E in the formula

$$k = Ze^{-\frac{E}{RT}},$$

is 25 k cal for reaction (1), 21 for reaction (2). In the case of reaction (1) we may calculate the constant k like in homogeneous solution, if we use large amounts of charcoal. E then turns out to have a value of 19 k cal ; a similar value of 20 k cal is found in homogeneous solution of methanol plus water. The retardation of the reaction in presence of charcoal increases with rising temperature. In reaction (2), on the other hand, the retardation decreases with rising temperature. This might be expected, if only the molecules in solution are reacting ; in homogeneous solution the temperature-coefficient is high, and, furthermore, the amount of adsorbed substance decreases with rising temperature, so a greater number of molecules are then in the interior of the solution.

It seems very likely that this retardation of chemical reactions on surfaces is of interest from the biological point of view.

Professor H. HARTRIDGE, F.R.S. : There are two conditions which must be satisfied when measuring the velocity of a reaction. One is that the reacting system must proceed from an unstable state to the position of equilibrium. The other is that measurements must be made of the concentrations of the reagents from time to time during this process. Our attention must first be directed to methods of satisfying condition one.

Methods of Causing Instability—There are several methods of displacing the chemical system into an unstable state :—

(1) By variation of one of the factors on which equilibrium depends, as, for example, the concentration of one or more of the reagents (rapid mixture method).
(2) By changing radiation (applicable to photo-chemical processes).
(3) By changing temperature ; and
(4) By changing pressure.

The time taken by the displacing procedure must be short compared to the time taken by the reaction to proceed to half completion. Some examples of these methods will now be given.

1. *The Mixing Method*—The mixing method has been applied to the reaction of gas mixed with gas, as, for example :—

$$C_2H_4 + Br_2 \rightarrow C_2H_4Br_2 \text{ (Stewart and Edland)},$$

also to the reaction of liquid mixed with liquid, as, for example :—

$$\text{dissolved } O_2 + HO \rightarrow O_2HO \text{ (Hartridge and Roughton)},$$

also to liquid mixed with solid, as, for example :—

dissolved O_2 + reduced red cells $\rightarrow$ oxygenated red cells

(Hartridge and Roughton).

The following might also be investigated by the mixing method : gas mixed with liquid and gas mixed with solid.

The mixing method may be applied in two ways, called the "stationary" fluid method, and the "moving" fluid method. In the stationary fluid method the fluids containing the reagents are intimately and suddenly mixed and enter a closed vessel in which the reaction then takes place. In the moving fluid method the fluids are mixed as above, but enter an open tube and travel down this with uniform velocity. The reaction occurs in this moving fluid. The stationary fluid method is usually inapplicable to fast reactions, to which the moving fluid method readily

lends itself. A diagram illustrating one of the apparatuses which Roughton and I have used during the last ten years was shown on the screen. The solution containing one reagent enters the apparatus and feeds the jets of odd numbers. The solution containing the other reagent also enters the apparatus and feeds the jets of even numbers, so that we have, say, four jets of odd number fed by one liquid, and four jets of even number fed by the other liquid. The liquids impinge on one another tangentially in a small mixing chamber, and very intimate rapid mixing takes place. The mixed fluids then proceed steadily down an observation tube, and as the diameter of the tube is known the rate of flow can be determined. Rates of flow up to 12 metres per second have been obtained. The success of the mixing apparatus depends on two factors : (1) rapid mixing, followed by (2) steady flow down an observation tube. In this tube the reaction proceeds so that, at any section of the tube, the reaction has reached a certain state.

It is interesting to note that the steady flow method was used by Rutherford in 1897 to measure the velocity of the loss of ionization by a gas. The gas having passed by an ionizing source, flowed steadily down an observation tube. At different points down this tube electrodes were placed, so that the residual ionization could be measured at these points. Since the time taken for the gas to travel from point to point down the tube was known, the rate of loss of ionization could be determined.

Where fluid is mixed with fluid, the fastest reaction that can be observed by the various mixing methods is as follows :—

Mixing Method	Time for half completion
By hand.	About 10 secs.
2-jet apparatus.	,, 1/100th sec.
16-jet apparatus.	,, 1/1000th sec.
High pressure apparatus.	,, 1/4000th sec.

The fastest reaction in fluids so far measured was one-half completed in 1/1000th of a second (Hartridge and Roughton).

2. *The Radiation Method*—The radiation method is applicable to photo-chemical reactions. In practice these are found to be of two kinds.

Type 1—Those in which ionization by light is required before the reaction can commence. For example, the reaction $H_2 + Cl_2 \rightarrow 2HCl$ cannot take place in the dark. Light must ionize the gases before the reaction can proceed.

Type 2—Those in which the position of chemical equilibrium in the dark is different to that when light is incident. For example, the equilibrium

$$COHb + O_2 \rightleftarrows O_2Hb + CO,$$

is shifted to the right in the light and to the left in the dark.

When measuring the velocities of such reactions, light is used to activate the components in type 1 and to displace the equilibrium of the components in type 2. For example, a solution of hæmoglobin in water containing also oxygen and carbon monoxide gases is caused to flow initially down a glass tube exposed to a powerful source of light, and subsequently down a glass tube in complete darkness. In the former the equilibrium suffers displacement from its initial position to the right, as shown in the above equation, and in the latter it returns towards the left to its initial position once more, and this velocity can be determined (Hartridge and Roughton).

3. *The Temperature Method* 4. *The Pressure Method*	These methods are theoretically possible, but, so far as is known, have not yet been put to practical use.

Methods of Measurement

Having considered the methods used for satisfying the first condition, namely, that the reaction must be caused to proceed, we must now briefly consider the second condition, namely, that measurement must be made, from time to time, of the concentrations of the reagents. This must be done moreover in a period of time which is short compared with that taken by the reaction to proceed to half completion. The following methods are available where rapid reactions are being studied :

Stationary Fluid method

Optical measurements, electrical measurements, pressure measurements.

Moving Fluid method

Optical measurements, electrical measurements, thermal measurements, chemical measurements.

Some examples of the latter may now be given :—

(1) *Optical measurements*—(*a*) Measurement of the position of absorption bands by means of reversion spectroscope (Hartridge) :—

$$\left.\begin{array}{r} Hb + O_2 \rightarrow O_2Hb \\ Hb + CO \rightarrow COHb \\ O_2Hb \rightarrow O_2 + Hb \\ COHb \rightarrow CO + Hb \\ O_2Hb + CO \rightarrow COHb + O_2 \end{array}\right\} \text{(Hartridge and Roughton, 1923.)}$$

(*b*) Measurement of light absorption by photo-electrometer—

$$\left.\begin{array}{r} O_2 + Hb \rightarrow O_2Hb \\ O_2 + Hcy \rightarrow O_2Hcy \end{array}\right\} \text{(Millikan, 1933.)}$$

$$\overset{-}{H} + H\overset{--}{CO_3} \rightleftarrows H_2CO_3 \rightleftarrows CO_2 + H_2O$$

(Brinkman, Margaria, and Roughton, 1933.)

(2) *Electrical measurements*—(*a*) Measurement of conductivity

$$\left.\begin{array}{l} \overset{+}{H} + H\overset{-}{CO_3} \rightleftarrows H_2CO_3 \rightleftarrows CO_2 + H_2O \\ \overset{=}{Cr_2O_7} + H_2O \rightleftarrows 2H\overset{-}{CrO_4} \end{array}\right\} \text{(Saal, 1928.)}$$

(*b*) Measurement of potential—

By Hydrogen electrode—$H_2CO_3 \rightleftarrows CO_2 + H_2O$ (Saal.)

By Antimony—Quinhydrone electrode—CO_2 reactions in blood. (Dirken and Mook, 1931.)

By Oxidation—reduction. (Ball and Chen, 1933. Saal.)

(3) *Thermal measurements*—Measurements made by thermo-couples either separately compensated or auto-compensated—

$CO + Hb \rightarrow COHb$ (Roughton, 1930.)

$Na_2Cr_2O_7 + 2NaOH \rightarrow 2Na_2Cr_8O_4 + H_2O$

(Lamer Read, 1930.)

(4) *Chemical measurements*—(*a*) By diffusion of dissolved gases out of moving fluid—

CO_2 reactions in blood. (Dirken and Mook, 1931.)

(*b*) By ultrafiltration of dissolved solids out of moving fluid --

CO_2, Cl, and O_2 reactions of blood. (Dirken and Mook, 1931.)

(*c*) By stopping reaction by mixing a third reagent with the two reagents after they have been allowed to react for a known time -

$CO + Hb \rightarrow COHb$ stopped by O_2 (Hartridge and Roughton, 1926.)

$H_2O_2 + Br_2 \rightarrow 2HBr + O_2$ stopped by Iodine in H_2SO_4

(Livingstone and Bray, 1928.)

$2\,NaNO_2 + H_2SO_4 \rightarrow 2HNO_2 + Na_2SO_4$ stopped by NaOH

(Schmidt, 1929.)

Whatever may be the method of measurement used it should have the characteristics of being applicable to a region of a moving fluid, which is narrow, at right angles to the direction of motion. The reason for this being that if the region is wide the measurements will average the values of parts of the fluid which have been reacting for different lengths of time.

Mr. G. Millikan : I should like to describe very briefly one particular form of the continuous flow method for measuring rapid reactions, of which Professor Hartridge has just been speaking. This technique, which many of you saw being demonstrated in the ante room, has been developed during the last few years at Cambridge to cope with rapid reactions occurring in various biological pigments. It is a lineal descendant of the Hartridge–Roughton technique, but has here been modified to satisfy the following two conditions : (*a*) a wide variety of colour reactions, and (*b*) very small amounts of material. The range of the method is now sufficient, we hope, to make it of some interest to those working in other fields than that of the blood pigments, for which it has been specifically designed. The essential principle remains as before: the two reacting fluids are driven—in the present apparatus by means of syringes connected to an electric motor—through two convergent tubes to a chamber, where they are thoroughly mixed together, and immediately passed down an observation tube, the extent of the ensuing reaction being observed at various points, each one corresponding to a known time after mixing.

The " colour " condition, imposed by the many different colour changes involved in reactions of the respiratory pigments and oxidation catalysts in which we were interested, has been satisfied by the development of

an extremely simple colour analyser, involving two colour filters and a rectifier type of photo-electric cell. Light from a suitable source passes through the observation tube transversely, and then through one of the two colour filters placed in front of the two opposing halves of the "differential" photo-cell. The filters are chosen so as to match the endpoints of the reaction being studied. If, for example, the reaction involves a colour change from red to green, red and green filters are used. When red fluid is in the observation tube, more red light will be transmitted than green, and more light will strike that side of the photo cell which is behind the red filter, giving a galvanometer deflection corresponding to this excess of light on one-half of the light-sensitive surface. When green fluid is in the tube, the conditions are reversed, and the galvanometer deflection is on the opposite side of the zero point.

The application of this method to blood pigments reveals a very happy collaboration between the biological creator of hæmoglobin and the physical chemical creator of the mercury arc, for the two absorbtion bands of oxyhæmoglobin in the yellow and green, which disappear on removal of the oxygen, come exactly at the wave-lengths where there are strong emission lines in the mercury arc spectrum; whereas reduced hæmoglobin absorbs much more of the purple line at 436 millimicrons than does the oxygenated form of the pigment (due to the shift in the very strong gamma band upon oxygenation). Filters are chosen, then, such that one of them lets through the purple and cuts out the yellow and green, the other absorbing in the reverse manner.

One of the advantages of the method is that, with reasonably dilute solutions, the calibration curve is linear, which means that there is an equal sensitivity throughout the entire range of the reaction. This can easily be seen theoretically; it has also been tested experimentally.

The second or "micro" condition manifested itself when we came to work on the blood of quite small or relatively valuable animals, from which only limited quantities of material was readily available. It has been satisfied (1) by the use of a very small observation tube (1 mm in diameter or less) and dilute solutions, together with a corresponding increase in the sensitivity of the measuring apparatus, an increase which was only possible because we were using a photo-electric cell method for following the reaction; and (2) by "quick-starting, quick-reading, quick-stopping" devices, by which measurements can be obtained before much fluid has been lost down the observation tube. With the present arrangement, we can get three points on a kinetic curve with an expenditure of 1/20 cc of blood in 5 cc of buffer solution, even when the reaction is taking place in 1/200th or 1/300th of a second.

The reduction (more properly the " disoxygenation ") of sheep hæmoglobin (shown in the last slide) will serve as a single illustration of the range and general accuracy of the method. The monomolecular character of the kinetic curve over the whole extent of the reaction is here brought out quite clearly.

Dr. F. J. W. Roughton : I should like to describe some quantitative applications of the rapid reaction velocity technique to (1) the testing of the law of mass action over time range 0·1 to 0·001 sec ; (2) the mechanism of chemical reactions (*a*) of the uni- or bi-molecular order, etc., or (*b*) for complex processes, the identification of constituent reactions ; (3) the rate of disappearance of transient, intermediate compounds, and (4) physiological problems.

Accuracy in estimation of the velocity constants is limited by the accuracy of observations and the character of the flow of the streaming fluid. The degree of accuracy is shown by the consistency of values in single experiments, and by a comparison with stationary methods where possible. The general accuracy in a single experiment is not better than 10%, but this is compensated for by the possibility of extreme variations in concentration of reagents. In some experiments we have got nearly a 100,000-fold variation in the time for half reaction, and find the process still conforming to the postulated kinetic equation.

I exhibit a study of the reactions of carbon dioxide in water. The rate of production of CO_2 when bicarbonate is mixed with acid is, over a wide range of p_H, proportional to the hydrogen ion concentration. These rapid reaction methods have been valuable for confirming over a much wider range than has been possible hitherto the validity of these mechanisms.

My next slide shows the results for velocity constants of a particular reaction. The various methods of estimation include manometric, indicators, conductivity, hydrogen electrode, and diffusion methods, all giving reasonably concordant results. The next example is that of a bi-molecular reaction, namely, the combination of carbon monoxide with hæmoglobin. If the reaction is simply bi-molecular, then, if we reduce the concentration of reagents, say, thirty-fold, the half-period should be thirty times longer. This is experimentally confirmed.

I now turn to a few examples of properties of unstable or transient compounds. The first one I take is an investigation by Ball and Cheng of the oxidation reduction potentials where the reductant is stable but the oxidant is unstable. That has been applied to a number of bio-

chemically interesting substances. The second example is the investigation of the ionization constant of acids, the molecules of which are unstable, such as carbonic acid, H_2CO_3. By the rapid method we arrive at a point on the true titration curve of the unstable acid before it has time to decompose, and in that way are able to calculate its true ionization constant. That has been done for carbonic acid. We hope to do it shortly for another unstable acid, namely, carbamic acid.

In another example of transient processes, a newly formed compound had a different reactivity. The process in question concerns the formation of reduced hæmoglobin from oxyhæmoglobin by mixing the latter quickly with sodium hydrosulphite. If the reduced hæmoglobin is at once mixed with carbon monoxide, it combines with the latter more quickly than if two seconds or more are allowed before the carbon monoxide reaction is brought about.

I come next to an example of a biological problem : to discover the rate at which carbon dioxide distributes itself between the plasma and the interior of a red blood corpuscle when CO_2 in the blood is increased. CO_2 migrates mainly in the corpuscles to start with, and there forms bicarbonate ions, and these come out in exchange for chloride ions from the plasma. Does that process go on quickly enough to serve in the actual circulation? Some data were shown on the screen for the rate at which carbon dioxide penetrates from the plasma into the corpuscles, and the rate at which bicarbonate formed therein escapes out into the plasma. The process takes a time of the order of a second or two, so that we have to take it into account in the very rapid processes which occur in the blood capillaries where times of this order are concerned.

The last problem I want to mention is an instance wherein these methods have been used to investigate both chemical reaction velocity and diffusion: the penetration of dissolved O_2 or CO into the red corpuscles of the blood. After the first molecules have entered, the next ones have to penetrate into a deeper layer in order to combine with hæmoglobin, and so we have a composite process involving not only a chemical reaction velocity, but also diffusion. That seems to be a condition we should expect to meet with often in biological processes, and therefore we should work it out in detail if we can. Attempts were made to calculate the rate of penetration of O_2 or CO into a layer of hæmoglobin solution of the same thickness as the corpuscle, but with no bounding membrane. The rate is found to depend on the thickness of the layer and on the square root of the velocity constant. Unfortunately, it is not possible to solve the equations for any but the earliest stages, but one can obtain a maximum solution and a minimum solution for the later stage, and the true

theoretical solution would have to lie somewhere in between these two. The diagram I show indicates the results of these calculations, and it will be observed that the two theoretical solutions are not too badly out. Thus one can get an idea of what the theoretical solution would be. The observed rate of penetration into the corpuscle is much slower, and a suggestion is that the difference between the theoretical rate for a layer of hæmoglobin solution and the observed rate for the corpuscle is due to the obstruction offered by the membrane which surrounds the corpuscle. That opens up the possibility that one may have by this combination of theoretical and experimental research a method of determining the diffusion constants of the actual membranes on the surface of cells.

In conclusion, I feel justified in claiming that these methods have already been of some service in biochemical and in biophysical problems, and I would like to urge that there must be many other opportunities for their use, both in biology and in pure physical chemistry.

Professor E. K. Rideal, F.R.S. : (A brief review was given, illustrated both by lantern slides and by the experimental apparatus of the results obtained using a new method of determining rates of reaction proceeding at interfaces.)

The method, based upon the views of the late Sir William Hardy about the structure of monolayers, consists in measurement of the rate of change in Volta potential at the interface. The work of Langmuir has shown that the orientation and sizes of the non-polar portions of a film-forming material can be examined by means of his well-known trough method, but information as to the nature or changes in orientation on compression of the polar groups is only indirect so far as such changes affect the relations of the non-polar portions to each other. Debye has shown that the polar and reactive groups of organic molecules are associated with a finite electric moment—the stray force fields of Sir William Hardy. Chemical reaction involves a change in the nature, and thus in the moment, of such groups.

In development of the method it was first shown that monolayers of film-forming materials changed the Volta potential at air water interfaces in a characteristic way, the extent of change being dependent on the nature of the head group. It was subsequently demonstrated that compression of such monolayers led to characteristic ΔV, n curves, which for many materials, on suitable substrates, revealed definite points of inflection ; these coincided with the points of inflection associated with changes of

phase of the film revealed in the Langmuir trough. The simplest example of interaction was next examined by the effect of alteration of the hydrion activity of the substrate in the phase boundary potential of several types of monolayers. The formation of oxonium compounds of ethers, alcohols, and acids with strong acids was investigated, and a typical neutralization curve of myristic acid film was revealed.

These preliminary investigations were sufficiently encouraging to warrant the application of the method to reactions actually proceeding in monolayers. The hydrolysis of γ-stearolactone was found to proceed quite smoothly on alkaline solution, and the reaction kinetics and energy of activation for the film reaction was found to be analogous to the bulk phase reaction, and to agree with Bronsted's formulation of such reactions. Incidentally, the rate of hydrolysis of palmityl chloride films on weakly alkaline solutions was found to be identical both on water and on 90% deuterium oxide.

One of the most important aspects of reactions in films is illustrated by the oxidation of films of unsaturated acids, such as oleic or petroselinic acids by dilute permanganic acid in the substrates. In extended films the reaction is unimolecular, but on compression of the film there comes a stage in the compression where the double bonds of the acids originally lying on the surface are raised into the hydrocarbon film. At this point attack by permanganic acid is controlled by the thermal agitation of the molecules, and it is possible to inhibit the reaction almost completely by suitable compression of the film. Alternatively, we may regard the reaction as analogous to the extension of muscle, *i.e.*, on extension of a film (suitably compressed) of oleic acid we can increase its rate of oxidation several hundredfold. It is also possible to "locate" the position of a double bond in a long chain by this method. The forces necessary to alter the configuration of the surface film, and thus the rate of reaction, are really surprisingly small, only a few dynes per cm, a fact which may not be without biological significance.

The method has been extended to the investigation of enzyme reactions, and all the usual bulk phase properties and kinetics can be repeated at interfaces. It has been found possible to prepare enzymes, "filmases," which appear only to attack proteins or lipoids, as the case may be, when these latter are in the form of monolayers. This last observation is opening up an interesting field of investigation.

The attack of a lecithin film by the lecithinases present in snake venom has revealed interesting features, for it has been found that the action of a 0·001% solution of black tiger snake venom on a film of lecithin which is normally attacked in a few minutes can also be inhibited by

suitable compression. Glyceride films on lipase solutions are likewise susceptible to an alteration in the molecular density of the film.

Finally, the effect of ultra-violet radiation on monolayers of such substances as ergosterol and of egg albumen can be examined with the greatest facility, and it is hoped that the results of an accurate photochemical investigation to obtain the exact quantum efficiency will be communicated shortly to the Society.

The speaker wishes to thank his colleagues and co-workers—Drs. Addink, Fosbinder, Gee, and especially Drs. Schulman and Hughes for the very active part they have taken in this investigation.

Professor J. B. S. HALDANE, F.R.S. : I should like to call attention to a type of measurement as far removed as possible from those described to us hitherto, namely, the measurement of what are generally known as subjective processes. A human being can estimate time by tapping at intervals believed to be of one second's duration, or by counting seconds. His temperature can be varied either by diathermy or as the result of fever.

The experiment has been made by François (1927)* and Hoagland (1933)†. In each case the rate of the process increases with temperature, the number of seconds in an estimated minute varying from 52·0 at 97·4° F to 37·5 at 103·0° F in one experiment. The results on a number of subjects agree with the theory that the process of time estimation depends on a reaction with a critical increment of 24,000 calories. It is worth noting that the effect of temperature on the pulse rate was far less regular. I wish to call attention to these experiments as illustrating the great variety of methods available for measuring reaction velocities.

Dr. M. POLANYI : I should like to submit some theoretical remarks on a group of reactions occurring in solutions. As the ultimate aim of this discussion is biological, this may be appropriate. A class of such reactions on which our ideas have been growing more distinct during the last few years is the substitution of negative groups by negative ions. A reaction of this class may be represented by—

$$X^- + RY = XR + Y^-$$

(X^- negative ion, $\quad Y$ negative group.
X negative group, $\quad Y^-$ negative ion.)

* François, M., 'Année Psychol.,' vol. 28, p. 186 (1927).
† Hoagland H., 'J. Gen. Psychol.,' vol. 9, p. 267 (1933).

Some examples which have been investigated are :—

$$\begin{aligned}
I^- + RCl &= IR + Cl^- \\
S_2O_3^{--} + RCl &= S_2O_3R^- + Cl^- \\
OH^- + RCl &= HOR + Cl^- \\
EO^- + RCl &= EOR + Cl^- \\
CH_3COO^- + RSO_2C_7H_7 &= CH_3COOR + {}^-SO_2C_7H_7
\end{aligned}$$

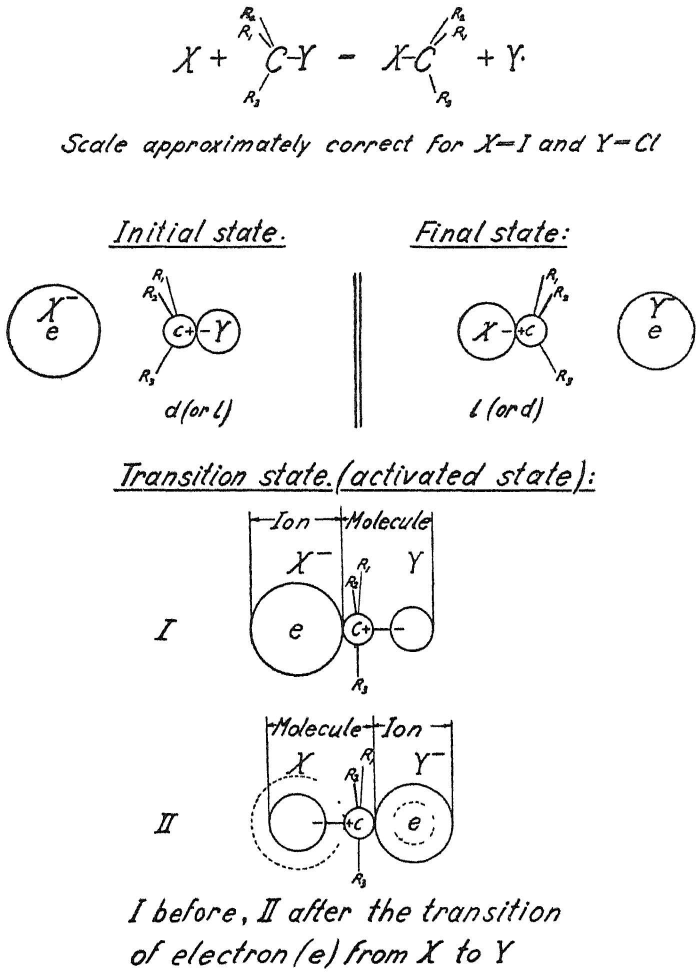


FIG. 1—Substitution by Negative ions

The idea of the mechanism of such reactions is illustrated by fig. 1. The negative ion X^- approaches the polar bond CY at its positive end. To facilitate the approach of the ion X^- the three substituents R_1, R_2, R_3,

which take no part in the reaction, move out of its way so that in the activated state the bonds linking them up to the central carbon atom lie approximately in a plane. A further important condition which is to be fulfilled if the reaction should take place is the elongation of the C–Y bond. This elongation is necessary to accommodate the increased size of the particle Y, which it attains when it is transformed into an ion Y^-.

This picture, which was first outlined in collaboration with N. Meer some three years ago,* has been brought into its present detailed form in recent work with R. Ogg in Manchester.†

In reviewing some conclusions reached from this picture, the first thing to be pointed out is that it contains no features which would point to a deviation from the bimolecular velocity law of Trautz and Lewis. Accordingly, it has been found by Moelwyn-Hughes‡ that the rate of all substitutions by negative ions can be expressed by the formula

$$k = Z\, e^{-\frac{Q}{RT}}$$

wherein Z is approximately 10^{10}, if k is expressed in mol/litre.

Furthermore, since the activated state is closely described in fig. 1, it will be expected that its energy Q can be at least approximately derived from the force constants which characterize the interaction of the atoms and ions involved in the process. In fact, Dr. Ogg and I hope soon to show elsewhere that often such calculations can be satisfactorily applied to experimental data. At present I wish to refer only to conclusions which can be drawn from our picture, without giving any calculations.

An obvious point is that some effort will be necessary to bring the three radicals R_1, R_2, R_3 into a plane, as required in our picture of the transition state. Large substituents R_1, R_2, R_3 should hence obstruct the approach of the ion X^- and form an obstacle to the reaction. They should cause a so-called " steric hindrance."

This is well borne out in a number of reactions where the action of negative ions on primary, secondary and tertiary compounds is found to become slower in the order primary, secondary, tertiary, in marked contrast to reactions of other types in which reactivity generally shows the reverse sequence.§

* Polanyi, " Atomic Reactions," Williams & Norgate, London (1932) ; ' Z. phys. Chem.,' B, vol. **19**, p. 164 (1932).

† ' Trans. Lit. Phil. Soc. Manchester,' vol. **41**, p. 41 (1934).

‡ " The Kinetics of Reactions in Solution," Oxford, 1933, p. 79.

§ See ' Z. phys. Chem.,' *loc. cit.*

It will be noted, furthermore, that it follows from the picture of the reaction that in its course the symmetry of the central carbon atom should be inverted. The mechanism of the reaction, indeed, fundamentally coincides with Werner's view of a substitution accompanied by Walden inversion.* We shall, therefore, expect a Walden inversion to take place every time a negative group attached to an asymmetric carbon atom is substituted by a negative ion.

This conclusion is again well borne out by facts. The regular occurrence of inversion accompanying substitution by negative ions has already been noticed by Holmberg.† Further cases have been adduced by Meer and by myself when putting forward the above views on the mechanism of ionic substitution. Some cases have been added by Wagner-Jauregg in his article on "Walden Inversion" in Freudenberg's 'Stereochemie.' This author even goes so far as definitely to recommend substitution by negative ions as a method of carrying out an optical inversion.

A specially interesting case arises when X = Y—that is, when we consider an interchange of chemically-equal particles :—

$$\begin{array}{ccc} R_2\ \ R_1 & & R_1\ \ R_2 \\ \diagdown\,| & & |\,\diagup \\ X^- + CX & = & XC + X^- \\ \diagup & & \diagdown \\ R_3 & & R_3 \\ d\text{ (or l)} & & \text{l (or } d) \end{array}$$

Such a reaction, if carried out with an optically active substance, will obviously lead to a racemic mixture of the two optically active forms.

Racemizations by negative ions have indeed been known for some time, as in the case of halogenated succinic acids, which were considered to be "catalytically" racemized by the corresponding halogen ions. Holmberg has even suggested that the racemization might be due to substitutions of the sort as suggested above. Other authors, however, considered the racemization as due to tautomeric change or other unknown causes.‡

* 'Werner Liebig's Ann.,' vol. **386**, p. 70 (1912).

† 'Ber. deuts. chem. Ges.,' vol. **59**, p. 125 (1926). I cannot see, however, that Holmberg's attempt to explain this fact (and Walden inversion in general) goes beyond a re-statement of Werner's mechanism.

‡ Wagner-Jauregg, 'Montsh. Chem.,' vols. **53-54**, p. 797 (1929). See also Hückel, "Theoretische Grundlagen der Organischen Chemie," Leipzig, 1931, vol. **1**, p. 258.

More recent work done in collaboration with Bergmann and Szabo has proved, however, that halogen ions can also racemize halogen compounds, in which no tautomeric changes are possible.

That these racemizations are in fact caused by an interchange of chemically identical particles is demonstrated by the homologous series of reaction rates shown in fig. 2. The rate of racemization of an optically active iodine compound caused by iodine ions is seen to fall into line with the rates of the chemical substitutions caused by other halogens. Other examples are in accordance with this result.

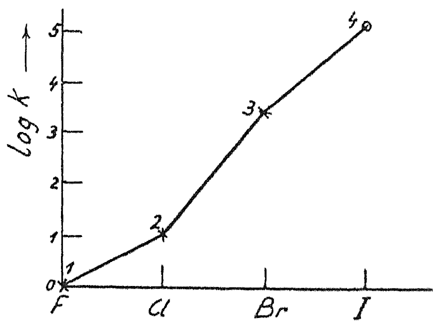


FIG. 2—Substitution of $CH_3-C(H)(C_4H_9)-X$ by I^-

(1)X = F; (2)X = Cl; (3)X = Br; (4) Racemization of $CH_3-C(H)(C_4H_9)-I$ by I^-. k velocity constant

Dr. McKeen Cattell: Professor Hill has indicated that the effects of increased hydrostatic pressures are important in relation to many biological phenomena. I have brought no data with me, and I shall speak only in relation to the effect of pressures on the contraction of muscle, a subject in which I have been particularly interested. When a muscle isolated from the body is immersed in a fluid, and that fluid is subjected to a pressure of the order of 200 to 300 atmospheres, there is a very large increase in the work done by the muscle. A single twitch may be increased from 50 to 100%, and in cardiac muscle the effects may be very much larger, perhaps four-fold. These changes are completely reversed with the release of pressure, and may be repeated many times.

It seems that this fact must be correlated with the influence of the pressure on the reaction velocities of the underlying chemical mechanisms. I am prompted to speak in this connection, having just received a paper by Fawcett and Gibson, of the Imperial Chemical Laboratories in Northwich, on the subject of their measurement of the reaction velocities of about fifty chemical reactions in the liquid phase. They have found, with only one or two exceptions, that pressures of the order of 3000 atmospheres produce an increase of from five-fold to ten-fold in the rate of chemical reactions. This effect must undoubtedly be related to the volume change, but the conditions there are obviously not simple. The effect on the reaction velocities is very much greater than the changes of volume, which would perhaps not amount to more than 5 or 10%. Perhaps the most important consideration in this connection is the fact that this change in volume would be confined to the spaces occupied by the molecules, and since the reaction velocities must be a function of the collision rate between molecules, it would be increased quite out of proportion to the volume changes.

I might mention, since we are interested in methods, that the use of pressure offers considerable possibilities for the study of biological processes. It can be applied almost instantaneously, and it rivals the methods of Roughton in the rate at which the biological material may be affected and the results recorded. It is possible, for example, to apply and release pressure during different stages of the muscle twitch, or in relation to other biological processes.

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Hypophysectomy of Birds

II—General Effects of Hypophysectomy of Fowls

By R. Towner Hill, National Research Council Fellow, A. B. Corkill, and A. S. Parkes, F.R.S.

(From the National Institute for Medical Research, London)

(*Received July* 6, 1934)

1—Introduction

It was originally supposed, mainly as the result of experiments on dogs, that the presence of the pituitary body was essential to life (see Cushing (1912) for discussion and references). Recent work, however, has shown that, in many species, the removal of the pituitary is not fatal. Thus dogs (Houssay (1932) and Houssay and Biasotti (1931)), cats (Allan and Wiles (1932)), and rabbits (Smith and White (1931)) show a fair survival rate, and rats (Smith (1930), Collip, Selye, and Thompson (1933)), and ferrets (Hill, M., and Parkes (1932)) a high survival rate after complete removal of the pituitary. Apart from regressive changes in the other endocrine organs, the most serious consequence of hypophysectomy appears to be disturbance of the carbohydrate metabolism. This disturbance varies in severity in different species, but in some it is responsible for at least a proportion of the deaths following hypophysectomy. Thus Houssay (1932) and Houssay and Biasotti (1931) consider that the anterior lobe of the pituitary is responsible for the formation of new carbohydrate from endogenous protein and that in the absence of the pituitary an animal is entirely dependent on pre-formed carbohydrate. This supposition is in keeping with the fact that hypophysectomized dogs occasionally develop a spontaneous fatal hypoglycæmia, while White (1933) observed that fatal hypoglycæmic convulsions were not uncommon among hypophysectomized rabbits. The spontaneous hypoglycæmia which may occur in dogs and rabbits after hypophysectomy is accompanied by a greatly increased sensitivity to insulin. This reaction has actually been used by Hartman, Firor, and Geiling (1930) as a test for the completeness of the operation. It is evident, therefore, that, while hypophysectomy cannot be regarded as a necessarily lethal operation in

species so far examined, an intensification of the resulting physiological disturbances might lead to a very high mortality.

The technique of hypophysectomy of the bird outlined in a previous paper (Hill, R. T., and Parkes (1934)) involves comparatively little damage, so that death from operative manipulation can almost be excluded. We were therefore surprised to find that most fowls died within two days of the operation. This was shown to be a genuine physiological effect by the fact that the mortality could be much reduced by appropriate therapy. The present paper deals with this post-hypophysectomy mortality in the fowl, and with the general effects observed in birds which were carried over the critical period and survived to show chronic pituitary insufficiency.

2—Material and Technique

All birds used were adult; the cocks were White Leghorns, Brown Leghorns, and Rhode Island Reds, but all the hens were Brown Leghorns, these being the most suitable for work on plumage changes following ovarian atrophy. The technique already described (Hill, R. T., and Parkes (1934)) was used without modification. The birds were allowed to feed heavily before operation, so that there was a full reserve of food in the crop. Blood samples were removed with a syringe from the superficial veins on the under side of the wings, or directly from the heart immediately after death. Blood glucose was estimated by the method of Hagedorn and Jensen.

The sellæ turcicæ of birds dying within 2 days were not examined microscopically unless there was some doubt of complete removal at the time of operation. The birds which survived beyond the first two days and subsequently died or were killed, were examined histologically for pituitary remains. Birds still living are showing the characteristic changes which we have been led to associate with complete removal of the anterior lobe.

Material available—Excluding three operations of a preliminary nature, and four on birds which were afterwards found to be diseased, 64 hypophysectomies on fowls are available for consideration. Seven proved to be incomplete. Six of the seven incompletely hypophysectomized birds were killed some weeks later when perfectly healthy, not having shown the slightest ill-effect from the manipulation. The seventh, a female, died 30 days after operation, by which time she was showing definite signs that the hypophysectomy had been at least partially effective. These birds are not considered further. Of the remaining

57, 30 were untreated after operation except that a few received saline or glucose, while the other 27 were treated with pituitary or adrenal cortex extracts.

Extracts used—The adrenal extract used was cortin (Allen and Hanburys' " Eucortone "). The pituitary extract was the alcohol-precipitated fraction of neutralized alkaline extract of acetone-desiccated anterior lobe. This powder was known to be gonadotropic, thyrotropic, and diabetogenic ; 1 gm = about 50 gm fresh tissue. 50–100 mg per day was given during the period of injection.

3—Hypophysectomy without Replacement Therapy

Survival Rate—The survival periods of the 30 birds untreated after operation is shown in Table I. Some of the earlier birds recorded in this table received glucose or saline, but this treatment was found to be ineffective and was abandoned. The others received no treatment whatever, except the one (HF 21) recorded as living 31 days. This bird was moribund and dying at 31 days, but was actually kept alive for another 13 days by injection of cortin.

Table I—Survival of Fowl after Hypophysectomy without Treatment

Days surviving*	Number of birds		
	Total	Males	Females
1	12	5	7
2	12	6	6
3	1	1	0
4	1	0	1
5	1	1	0
10	2	1	1
31	1	0	1
Total	30	14	16

* " Surviving 1 day " means that they were found dead or that they died on the day following the operation; " surviving 2 days " refers to deaths recorded on the 2nd day after the operation, etc. Actually, therefore, " 1 day " is about 12 hours–36 hours, etc. No birds which successfully recovered from the anæsthetic died on the same day. Anæsthetic deaths during the operation amounted to about 10%.

From this table it may be concluded that without appropriate treatment 80% of hypophysectomized fowls die within 48 hours. Some will live for a few days, but survival for a period of weeks is rare. No difference in the survival of cocks and hens was observed.

The behaviour of the birds before death is usually well defined. For 6–18 hours after operation they are bright-eyed, active and quite willing to eat. They then become progressively more sluggish, with eyes dull and half-closed and head and tail drooping. They drag their feet, and the walk degenerates into a waddle. Later they pass into semi-coma. Four out of six birds actually observed at the time of death died in weak to moderate convulsions. Birds living for more than 48 hours died after a long period of increasing moribundity characterized by inactivity and loss of appetite. Forcible feeding was tried, but without success, as the material merely stayed in the crop. Inanition may have played a part in the later deaths, but cannot have been a factor in those occurring within 48 hours.

Blood Glucose Level—In view of the work of Houssay (1932) and Houssay and Biasotti (1931) on the dog, and that of White (1933), and

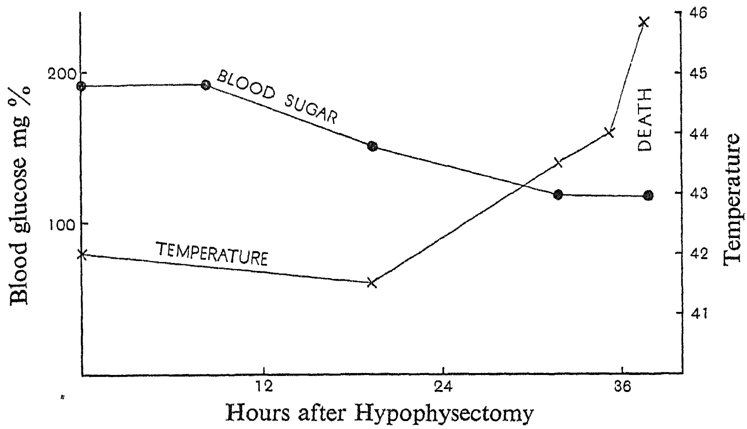

FIG. 1—Blood glucose and body temperature changes after hypophysectomy in Brown Leghorn hen (HF 53)

Corkill, Marks, and White (1933) on the rabbit, it seemed possible that the wholesale mortality was due to disturbance of the carbohydrate metabolism. This supposition was confirmed to some extent by estimation of the blood glucose in some of the later birds receiving no treatment whatever. Thus in one of the birds dying 10 days after operation (HF 57), the blood glucose was 163 mg % on the day after operation and 124 mg % 4 days later, as against the normal value of about 200 mg given by the Hagedorn and Jensen method*. Another bird dying at $21\frac{3}{4}$ hours after hypophysectomy (HF 56) showed a blood glucose of 152 mg %.

* As other observers note, fowls have a blood glucose level, estimated by reduction, approximately twice that usually found in mammals. About 15% of this reduction value is due to non-carbohydrate substances such as ergothionene.

A series of observations made on an uninjected bird (HF 53) which died $37\frac{3}{4}$ hours after operation gave the results shown in fig. 1. In this bird the blood glucose dropped from 191 mg % at operation to 119 mg % 32 hours later. These results, together with those on treated animals recorded below, suggest that the blood glucose begins to drop between 8 and 12 hours after operation and undoubtedly indicate some disturbance of the carbohydrate metabolism. It is known, however, that blood glucose values such as these are perfectly compatible with life in fowls, and our own later results show that the observed drop in blood sugar cannot have been a major factor in the high mortality in the first 48 hours after hypophysectomy. Certainly the convulsions which were observed in some of the birds at death cannot be considered as indicating hypoglycæmia, because the severest observed were in a bird with a blood glucose of 177 mg %, and, in any case, extreme hypoglycæmia in normal fowls given insulin is not accompanied by convulsions.

Body Temperature—The most remarkable symptom observed to precede death in the first 48 hours was a rapid rise in body temperature. The maximum temperature range we have noted in normal fowls has been $40\frac{1}{2}$° C–42° C. HF 53 had a temperature of 44° C, three hours before death and of nearly 46° C at death (fig. 1). HF 56 rose from $41\frac{1}{2}$° C at operation and 42° C at $17\frac{1}{4}$ hours later to $45\frac{3}{4}$° C at death $21\frac{3}{4}$ hours after operation. HF 57, which died 10 days after operation, showed no signs of increased temperature 24 hours before death, so that it is obviously an immediate pre-mortem symptom.

4—Hypophysectomy with Replacement Therapy

None of the birds which died gave any suggestion that the actual operative manipulation was a contributory cause of death. One or two developed mucous obstruction of the glottis, but it is very doubtful if this was an effect of the manipulation, especially as it was not found in incompletely hypophysectomized birds. This fact, together with the symptoms observed to appear before death, makes it certain that the high mortality in the first two days is a genuine physiological effect of the removal of the pituitary, but at present we are quite unable to offer any sufficient explanation for it.

The next step seemed to be to find some method of keeping the birds alive. This was the more necessary, in that the primary object of performing hypophysectomy in the fowl was to study the subsequent changes in the reproductive organs and secondary sexual characters, and the results described above showed clearly that no headway would be made

until some method of prolonging survival could be found. Since administration of glucose had proved ineffective, it seemed that replacement therapy would have to be undertaken. Pituitary extract would presumably retard the changes in the reproductive organs, and since the well-known effect of hypophysectomy on the adrenals might be one of the more serious results of the operation, it was decided to try giving cortin. It was scarcely likely that cortical insufficiency would develop in 24 hours, and the symptoms observed before the early deaths were not those characteristic of cortical deficiency, but there seemed a bare possibility of obtaining an improvement.

Survival with Cortin—Twelve birds were given 1 cc "Eucortone" twice a day for four days beginning immediately after the operation. The results are shown in Table II. It will be seen that only three birds died while cortin was being given, though four more died in the three days after the last injection. The remaining five survived.

Two were killed at 15 days after operation, one of them being in bad condition. Another (HF 37) was killed in excellent condition for histological material at 5 weeks, while the remaining two are still alive and in good condition (June 12, 1934). It appears, therefore, that the administration of cortin had materially assisted the birds over the first few critical days, and that subsequently a large proportion were able to carry on without further assistance. This conclusion is so unlikely that we do not regard it as anything more than suggestive, but the fact remains that whereas 30 untreated hypophysectomized birds yielded one alive, and that in poor condition, a fortnight after the operation, 12 cortin-treated hypophysectomized birds yielded five alive at this time, four of which were in good condition. Of the six males given cortin, four survived more than 2 weeks. Of the seven females only one. On these figures, therefore, there appears to be a sex difference in survival, but the numbers are only small. Our limited experience suggests that fowls of a heavy breed survive better than those of a lighter one. Cortin also appeared to have some effect in relieving chronic pituitary insufficiency in HF 21. This bird was the only one which survived the first fortnight without treatment. At 31 days after operation it was moribund. Three daily injections of 1 cc "Eucortone" improved its condition greatly. A week later it was sinking again. Life was further prolonged for 5 days by cortin injection, but the bird was very ill when killed the following day.

Survival on Anterior Lobe Extract—Since the results with cortin suggested that a few days' treatment over the critical period would enable the survival rate to be raised to a satisfactory point, it was decided to see

Table II—Effect of Cortin and Anterior Lobe Extract on Survival of Fowl after Hypophysectomy

No. of bird	Sex	Days surviving	Treatment for the first 4–6 days after operation only	Notes
HF 44	♀	1	Cortin	
43	♂	3	,,	
49	♀	3	,,	
45	♀	4	,,	
48	♀	4	,,	
38	♂	5	,,	
46	♀	6	,,	
51	♀	15	,,	Killed ; in bad condition.
42	♂	15	,,	Died after castration ; in good condition.
37	♂	35	,,	Killed ; in excellent condition.
40	♂	60+	,,	Living and in good condition June 12, 1934.
36	♂	64+	,,	Living and in good condition June 12, 1934.
59	♀	1	Anterior lobe extract	
61	♀	1	,,	
62	♀	1	,,	
67	♂	2	,,	
69	♂	2	,,	
63	♀	6	,,	
70	♂	14+	,,	Living and in good condition June 12, 1934.
71	♂	14+	,,	Living and in good condition June 12, 1934. Injections continued.
54	♂	15	,,	
68	♂	15+	,,	Living and in good condition June 12, 1934.
64	♀	16	,,	
65	♀	20+	,,	Living and in good condition June 12, 1934.
66	♀	20+	,,	,,
60	♀	34+	,,	,,
52	♀	43+	,,	,,

whether pituitary extract would be effective (cortin is notoriously expensive and a few days postponement of the genital atrophy would be immaterial). Fifteen hypophysectomized fowls have been given pituitary extract immediately after the operation for 4–6 days. Nine of these have lived

more than a fortnight and seven are in good condition at the time of writing (June 12, 1934).

Behaviour on Cortin and Anterior Lobe Extract—Taking the 27 treated fowls together, there is certainly a significant increase in the survival rate as compared with the untreated ones. Fourteen of the 27 treated birds survived more than a fortnight as compared with one of the 30 non-treated animals. It is very noticeable that birds carried over the critical period by treatment do not show the characteristic immediate post-hypophysectomy symptoms mentioned on p. 211. If they die subsequently the symptoms appear before the end, but in a less acute form. Birds in which prolonged survival follows the treatment maintain fair condition and show no ill-effects except that the appetite tends to be fitful. Those dying during the critical period in spite of injection show the usual symptoms. Thus HF 61 (anterior lobe extract treated) had a temperature of 45° C 30 minutes after death.

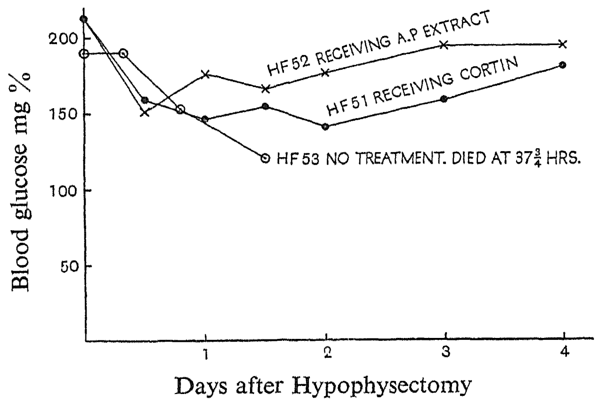

FIG. 2—Blood glucose level changes after hypophysectomy in Brown Leghorn hens receiving no treatment, cortin, or anterior lobe extract

Blood Glucose Level during Cortin Injection—Blood glucose records during cortin injection are available for only one bird (HF 51). These are given in fig. 2, and, so far as they go, suggest that the initial post-operative drop in blood glucose level is not materially affected. The rise towards the end of injection and the subsequent drop may be significant, but HF 21 had a blood glucose of only 56 mg when killed 44 days after hypophysectomy following 5 days cortin injection.

Blood Glucose Level during Anterior Lobe Extract Injection—The blood glucose of four birds, Nos. 52, 59, 60, 61, receiving anterior lobe extract injections was in each case taken on the day following the operation. The values obtained were respectively 177, 150, 178, and

199 mg %. The first and the third of these animals survived 43+ and 34+ days while the second and fourth died almost immediately. Three of these values are above those obtained at a corresponding time for untreated animals and some effect of the extract may be indicated. The subsequent increase in the blood glucose level of HF 52 during the period of injection (fig. 2) is certainly significant. A clear result was obtained on HF 46 in which the blood glucose level had remained at about 140 mg % from 10 a.m. to 6 p.m., but following an intravenous injection of anterior lobe extract was found to be 177 mg % when it died at 11.40 p.m. It may be noted that these results provide further evidence that the low blood glucose level is not a primary cause of the mortality of the hypophysectomized fowl.

5—Effects of Chronic Pituitary Insufficiency

There are three untreated hypophysectomized birds which lived more than 1 week after operation and 13 which lived more than 1 week after the last injection of cortin or anterior lobe extract. We have made certain observations of a general nature on these birds which may suitably be recorded here.

Body Weight—Adequate weight curves are available for 10 birds after hypophysectomy; these are given in figs. 3 and 4. All the birds received either cortin or anterior lobe extract during the first few days after hypophysectomy. There is a loss of weight either immediately, or else after a 2–3 week period of comparative stability. This loss is afterwards retrieved if the bird lives. Closer examination of these curves is interesting. HF 36 and 37 were Rhode Island Red cocks receiving identical treatment for the period covered by the curves. Both show a slight gain in weight over the first fortnight, followed by a loss and again by an increase. HF 37 was killed at 5 weeks for histological material when in splendid condition, the net change in weight since the operation being 60 gm. Half this loss could have been accounted for by the atrophy of the testes, which weighed under 1 gm! HF 40 and 54 were Brown Leghorn cocks. Both showed an initial decline in weight, HF 54 dying after 15 days. HF 40 recovered from the initial loss but afterwards declined again during a temporary lapse (see below). HF 51, 52, 60, 64, 65, 66 were Brown Leghorn hens and the curves are very similar, except that HF 52 stabilized early. It is evident that when the birds have become stabilized after hypophysectomy there is not necessarily any serious loss of weight.

Body Temperature—In birds which have survived the immediate post-hypophysectomy period we have observed no regular disturbance of the body temperature. HF 54 had a temperature of 38° C at death, but having not eaten for some days, was in a condition of partial inanition. HF 40, at the worst period of its excessive moult, suddenly began to lose temperature, which dropped from 38° C to 31° C in 13 hours. The bird was moribund at the lower temperature. This suggested a possible cortical deficiency, so 2 cc cortin was given intramuscularly and the cage was enclosed and heated to about 30° C. As a result of one or both of these treatments the bird recovered in a remarkable manner, and three

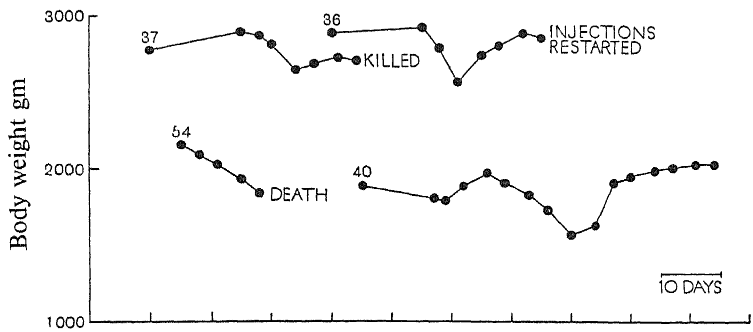

FIG. 3—Body weight changes in cocks after hypophysectomy

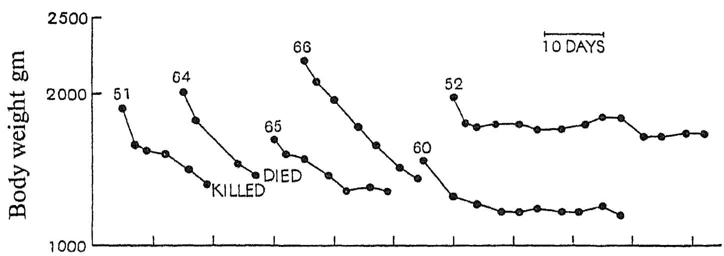

FIG. 4—Body weight changes in hens after hypophysectomy

days later had gained normal temperature and activity. Apart from these two birds we have no record of abnormal temperature after the first week post-operation.

Moulting—Moulting after hypophysectomy was reported by White (1933) in rabbits and has also been noticed in ferrets. All hypophysectomized birds surviving more than a fortnight have shown signs of moulting, particularly HF 40, a cock, and HF 52, a hen. The former became almost bare.

Plumage—Selected areas of the birds were plucked at the time of operation or soon afterwards to study the plumage changes following

hypophysectomy. These will be dealt with separately, but it is relevant here to record that both in plucked and in moulted areas, the regeneration of feathers proceeds at about the normal rate. After plucking, for instance, the regenerating breast feathers were observed to break through the skin in about 10 days. So far, we have observed no obvious changes in the new plumage after hypophysectomy except such as can probably be referred to the atrophy of the gonads and thyroids, which would be expected to follow hypophysectomy.

Pugnacity—Although the cocks lose their characteristic carriage and become less provocative, they will still fight and it has been found necessary to keep them separate even after the testes have completely atrophied.

Blood Glucose—The blood sugar levels in five birds which survived the operation for a comparatively long period are given in fig. 5. These suggest that when the bird has become stabilized, the blood glucose level is relatively constant at a point rather below the normal. On the

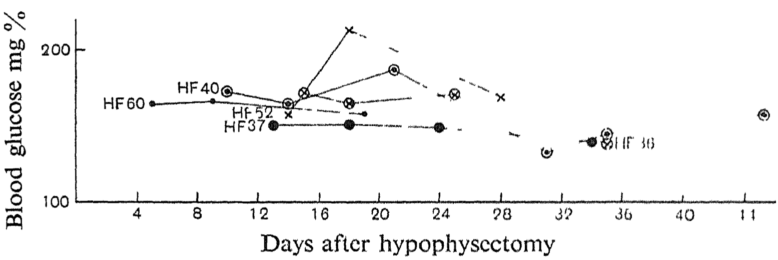

FIG. 5—Changes in the blood glucose level in fowls surviving the last injection for more than 1 week

other hand, decline and death during the second weeks seem to be associated with a low blood glucose. Thus HF 51 had a value of 115 mg % 14 days after operation and one day before she was killed in bad condition; HF 54 showed only 54 mg % when dying 15 days after operation; and HF 57 (an untreated animal) showed 124 mg % five days before death and five days after operation.

Sensitivity to Insulin—In comparison with mammals, the fowl is extremely resistant to insulin (Cassidy, Dworkin, and Finney 1926). With relatively large doses the blood glucose falls, but tends to recover its original level after a few hours. Even with enormous doses, convulsions or muscular twitchings are not observed and the only symptoms associated with the hypoglycæmia may be a slight unsteadiness and disinclination to move about. These symptoms disappear as the blood glucose level rises.

In view of the fact that hypophysectomized dogs and rabbits show a greatly increased sensitivity to insulin it might be thought that the hypo-

physectomized fowl would behave in a similar manner. As a control, we gave a normal hen 50 units of insulin ; the blood glucose was reduced from 200 mg % before injection to 106 mg % $1\frac{1}{2}$ hours later (the maximum drop observed) but neither then nor subsequently did she show signs of distress. HF 37 was given 10 units 35 days after operation without obvious effect; the blood sugar at $2\frac{1}{2}$ hours was 90 mg % against a reading the previous day of 139 mg. HF 51, although in bad condition, gave no indication of being affected by 5 units 14 days after operation or by 10 units the following day. We may conclude therefore that hypophysectomized fowls do not show the greatly increased sensitivity to insulin found in dogs and rabbits.

6—Discussion

We consider that the results described above establish that hypophysectomy is almost a lethal operation in fowls. The operative manipulation is not a contributory factor; incompletely hypophysectomized birds survived the operation and administration of cortin or anterior lobe extract greatly reduced the mortality after complete removal of the pituitary. It is difficult to offer any adequate explanation of the high mortality in the first 48 hours. For the following reasons, the fall in blood glucose after the operation was not a primary factor:—

(*a*) A much greater fall can be caused in the intact fowl by insulin without adverse symptoms.

(*b*) Glucose had no effect in lowering the mortality.

(*c*) Birds carried over the critical post-hypophysectomy period lived in good condition with a blood glucose much lower than that observed in many dying during the early period.

(*d*) One fowl died in convulsions when the blood glucose had been raised from 140 mg % to 177 mg % by an intravenous injection of anterior lobe extract.

It seems most improbable that atrophy of the other endocrine organs after hypophysectomy, which probably happens in the fowl as in mammals, could have fatal effects in so short a period. We deduce, therefore, that there is some severe metabolic disturbance. With this is associated a pronounced lowering of the blood glucose, but not to a level so low as to have by itself a lethal action.

We are unable to offer an explanation of the apparent efficacy of cortin in carrying the animals over the critical period.

7—Summary

Fifty-seven fowls hypophysectomized by the method previously described are discussed in relation to survival and effects of chronic pituitary insufficiency.

Of 30 which received no treatment after operation (except that a few had glucose) 24 or 80% died within 48 hours of operation ; only 1 survived more than 14 days. The symptoms preceding death included a sluggishness increasing to semi-coma, a fall in blood glucose and a remarkable rise in body temperature. Mild convulsions at the time of death were observed in some fowls.

Twenty-seven were given cortin or anterior lobe extract for 4–6 days, starting immediately after operation. Of the 12 receiving cortin only one died within 48 hours and 5 survived more than a fortnight. Of the 15 receiving anterior lobe extract, 9 survived more than a fortnight. Birds dying during the critical 48 hours in spite of injection exhibited the same symptoms as the untreated ones. Those surviving showed a gradual recovery in the blood glucose level during the time of the injections and exhibited no adverse outward symptoms.

The general effects of chronic pituitary insufficiency, as observed on birds living for more than 1 week after operation or after the last injection of anterior lobe extract, include a drop in body weight which may be recovered later, heavy moulting, some loss of activity and a decreased blood glucose, but no increased sensitivity to the effects of insulin.

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Hypophysectomy of Birds

III—Effect on Gonads, Accessory Organs, and Head Furnishings

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(*Received July* 27, 1934)

[Plates 5–8]

1—Introduction

It is now well known that removal of the anterior lobe of the pituitary body in mammals leads to atrophy of the gonads and thence of the accessory reproductive organs,* but this generalization has not hitherto been extended to birds. It would be expected that hypophysectomy would result in atrophy of the secondary sexual characters directly dependent on the gonad. Such characters are rare in experimental mammals, but the comb of the male fowl is an obvious example.

The present paper records observations on the changes in the testis and vas deferens, in the ovary and oviduct and in the head furnishings of birds after removal of the pituitary body. Plumage changes, which do not depend on the testes and are only indirectly linked with the ovary, are not included.

2—Technique

The operations were carried out as described previously (Hill, R. T., and Parkes (1934)). The completeness of the removal of the anterior lobe has been checked histologically on all dead birds recorded in this paper.

Comb Measurements—The measurements were made with callipers, the length being the greatest antero-posterior extent and the height taken from the middle of the root on the head to the top of the highest point immediately above. During atrophy the points of a large comb tend to rotate forwards, but so far as possible measurements were made to the same point. Flaccid combs, chiefly in hens, were straightened before

* It seems unnecessary to give references to the rapidly growing literature on this subject.

measuring. The best index of the size of comb would, of course, be the volume, but we have found no satisfactory method of estimating the very variable thickness and we have therefore used length × height as an expression of the size. This still underestimates the actual size changes, which, of course, are three-dimensional, but it gives a fair index for the surface area and a much better impression of what is actually happening than length alone, or even length + height. The hanging diagonal length of the right wattle was taken.

Histological Technique—This has been as usual. The size of testis tubules is expressed as the average diameter of 10 tubules measured at right-angles to the long axis.

Experimental Material—The histological material is not extensive, as we have naturally preferred to keep the birds for studying the possible plumage changes after hypophysectomy, rather than kill them to study the more certain gonad changes. Six cocks (HF 12, 19, 37, 42, 54, and 70), which were fully adult and apparently in full sexual activity at operation, died or were killed between 5 and 35 days after hypophysectomy; of these only two were given anterior pituitary extract for 4 to 6 days after operation. Two others received cortin, but this can scarcely have affected the reproductive organs. The six cocks form a fairly good series. Comb measurements are also available for four cocks (HF 36, 40, 68, and 71), still living.

Four hens died or were killed at various times between 15 and 44 days after operation. We have not thought it worth while to include earlier stages. Of the four, two received anterior lobe extracts for the first five days after operation and one received cortin over a similar period. The one living 44 days received two series of cortin injections in the last two weeks. They are a less satisfactory series than the cocks, because the reproductive organs of the normal hen vary greatly according to whether or not she is laying heavily; and although two of the four were known to be laying at the time of operation, we cannot be sure that they would have continued to do so. In addition to the four hens available for histological examination, three others still alive have provided comb measurements.

Stages of testis atrophy were also obtained from one turkey, one Sebright bantam and two pigeons.

Control Material—The testes of 10 normal adult cocks, mostly Rhode Island Reds and Brown Leghorns, serve as control material. The weight of these varied from 7·4 gm to 35·9 gm the pair, the average being 20·5 gm. All were in full spermatogenesis. The average tubule diameter measured in one of these was 266 μ, fig. 14, Plate 7. The vas deferens

from one of these birds was sectioned, and found to be crowded with sperm ; the average diameter above the swelling at the distal end was 1·5–2 mm. Histological examination of the combs of normal cocks showed the typical condition described by Hardesty (1931).

The size of the hen ovary during activity varies greatly, of course, according to the number and size of the yolks present. Ovaries from two hens in full lay, with eggs in the oviduct, weighed 31 and 50 gm. The oviducts were about 50 cm long. A number of non-laying hens had ovaries of 2–5 gm and oviducts 15–20 cm long. The normal histological appearance of the oviduct of a laying hen, in the region of the albumen gland, is shown in fig. 21, Plate 8.

3—Effect on the Male Fowl

Behaviour—Most of the cocks have crowed occasionally during the first week after operation, and some during the second week. Only one (HF 40) was heard to crow later than this, on the 17th day after removal of the pituitary. Interest in hens wanes over the same period; some attempts at " treading " were seen at the end of the second week, but soon afterwards the behaviour becomes similar to that of the capon.

The Testes—The earliest post-hypophysectomy testes available are from HF 12, which died five days after operation. The weight of these testes (Table I) is just below the range observed for normal cocks. The testes of the other five birds showed quite definite regression. Those of HF 37, 35 days after operation, were remarkable, being very small and yellow externally. The fresh testes on cutting were seen to be brown inside and of the consistency of stiff jelly, no tubules being visible. The tunica was extremely thick. Fig. 1 shows that the testes of HF 37 were probably approaching their minimum weight.

As may be seen from the tubule diameters given in Table I, the testes of HF 12, 19, 42 and 37, form a well-graded series histologically. There was still an abundance of spermatids and spermatozoa in HF 12 (5 days after hypophysectomy) but the tubules were already looking disordered. HF 19, at 10 days, had spermatozoa only in occasional tubules, while in many tubules spermatocytes were the most advanced stages persisting. The intertubular tissue, however, which is sparse in the normal fowl, was not visibly increased in relative amount. The testes of HF 42, at 14 days, were much more degenerate, fig. 15, Plate 7. The lumen of the tubules was almost occluded by syncytium, and no later stages than spermatocytes, mostly primary, were observed. The intertubular space was still not relatively greater than normal, but the tunica

albuginea had become much thicker, presumably as a result of contraction. In the testes of HF 37, five weeks after hypophysectomy, the tubules had shrunken to about one-fifth of the normal diameter and consisted only of basement membrane, a ring of spermatogonia surrounding a syncytial mass and a few Sertoli cells, fig. 16, Plate 7, and fig. 20, Plate 8. These tubules were comparable with those found after X-ray sterilization of a mammal. The intertubular tissue occupied more than half the sectional area ; it was largely fibrous, the glandular cells

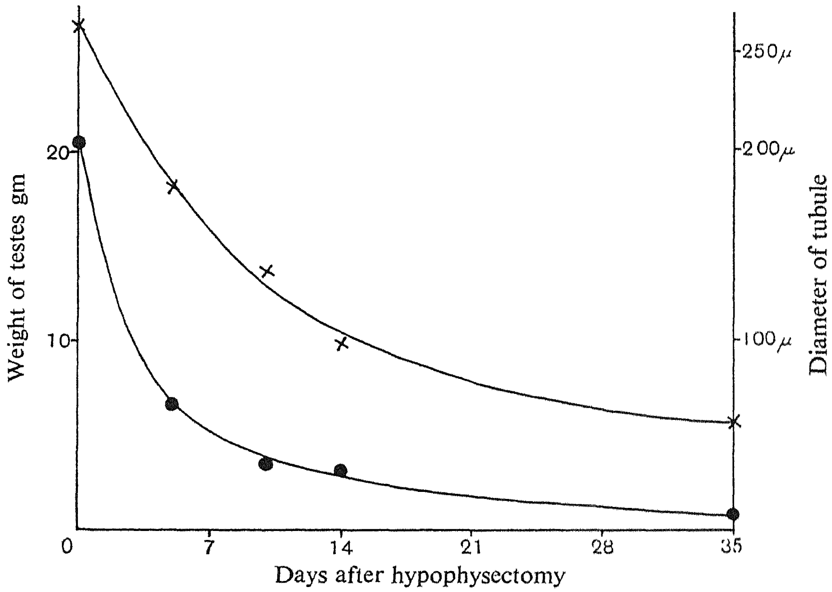

FIG. 1—Atrophy of fowl testis after hypophysectomy. ● weight of testis ; × diameter of tubule

being small and inactive. In this respect, of course, it differed greatly from that found after X-ray sterilization, when there are masses of well-developed glandular cells. The testis of the hypophysectomized fowl differs from that of the hypophysectomized ferret (and probably other mammals) in showing a greater shrinkage of the tubules and a greater relative increase in the intertubular tissue.* The testes of HF 37 were, in fact, the most degenerate testes which we have ever seen. The shrunken tunica was extraordinarily thick, about 150–250 μ, as compared with 20–25 μ for the normal testes.

The tubules of HF 54 were large compared with those of HF 42, taken at a similar stage after operation, and they contained spermatozoa.

* It is scarcely necessary to emphasize that this apparent increase in the amount of intertubular tissue is purely relative to the size of the tubules.

TABLE I—Hypophysectomized male fowl

No. of bird	Temporary treatment after hypophysectomy	Time of last observation* after hypophysectomy (days)	Testes		Comb				Wattle length		Notes
					At operation		At last observation*				
			Weight (gm)	Diameter of tubules (μ)	Length (cm)	Height (cm)	Length (cm)	Height (cm)	At operation (cm)	At last observation* (cm)	
HF 12	None	5	6·6	181	12·0	7·2	—	—	7·3	—	Died.
19	,,	10	3·5	137	10·2	5·5	—	—	7·6	—	,,
42	Cortin	14 (castration)	3·1	88	9·2	4·6	7·7	4·0	5·7	4·2	Died 1 day after castration.
54	Anterior lobe ext.	15	1·9	155	11·5	7·9	9·8	6·5	8·2	6·4	Died.
37	Cortin	35	0·8	57	9·0	—	6·1	—	6·1	3·5	Killed — condition very good
36	,,	81 (still living)	—	—	9·9	4·6	6·5	2·9	7·1	4·2	Used for replacement experiments after 35 days.
40	,,	77 (still living)	—	—	10·3	6·9	6·0	2·8	7·4	3·8	
68	Anterior lobe ext.	32 (still living)	—	—	13·2	7·2	8·6	4·5	7·5	5·4	
70	,,	20	2·5	97	12·9	6·8	11·5	5·9	9·3	7·2	Died.
71	,,	31 (still living)	—	—	14·0	8·5	9·6	5·3	8·3	5·3	A.P. injection continued all through.

* " Last observation " refers to time of death, killing or latest observation on those still living (June 29, 1934).

This difference is probably due to the fact that HF 54 received anterior lobe extract for 5 days after operation, while HF 42 did not. The testes of HF 70, also receiving anterior lobe extract for 5 days after operation, contained no later stages of spermatogenesis than spermatocytes, but they were also less atrophic than would have been expected from the series not receiving anterior lobe extract.

Vas deferens—Figs. 24 and 25, Plate 8, show the contrast between the vas deferens in a normal fowl and in HF 37. The most obvious difference is one of size, the operated bird having a vas only about one-fifth the diameter of that of the normal. There are also differences in the lining epithelium. In the normal cock, this is high columnar, the basal nucleus in each cell leaving a clear cytoplasmic border next to the lumen. The epithelium is ciliated. In HF 37, the epithelium of the vas is low cuboidal, the cells having little cytoplasm and scarcely any cilia, figs. 24 and 25, Plate 8. The vas of HF 54, 15 days after hypophysectomy, is at an intermediate stage. Neither vas contains obvious spermatozoa, in contrast to that of the normal cock. In mammals, spermatozoa are found in the epididymis for some weeks after hypophysectomy since they are able to survive there for a long time. In view of our findings on the controls, it is most improbable that both HF 37 and 54 were infertile at the time of operation (HF 54 actually had a few spermatozoa in the testis at the time of killing) and it must be concluded that the power of retaining existing spermatozoa is less in fowls than in mammals.

Comb and Wattles—Comb measurements are available for HF 42, 54, 70, 37, 36, 40, 68, and 71. Of these HF 68 and 71 were used for a maintenance experiment and need not be considered here, while HF 42 and 54 and 70 died before regression was far advanced (see Table I). The comb of HF 37 was damaged and only length measurements were made, but good figures were obtained for HF 36 and 40.

The comb of HF 42 decreased from $9 \cdot 2 \times 4 \cdot 6$ cm at the time of operation to $7 \cdot 7 \times 4 \cdot 0$ cm at the time of killing, an area decrease of 25%. The comb of HF 54, injected with anterior lobe extract for 5 days after operation, decreased from $11 \cdot 5 \times 7 \cdot 9$ cm to $9 \cdot 8 \times 6 \cdot 5$ at death 15 days after operation, an area decrease of 27% ; that of HF 70, receiving similar treatment, had regressed 23% at death 20 days after operation; HF 37's comb was $9 \cdot 0$ cm long at operation and $6 \cdot 1$ cm long when the bird was killed at 5 weeks after operation. The regression of the combs of HF 36 (Rhode Island Red) and 40 (Brown Leghorn) is shown in fig. 2, expressed as length $\times$ height values. These curves show that regression is rapid and continuous until an area of about one-third the original is reached, when a second phase of regression sets in, characterized

by slow shrinkage of the remains. By analogy with castrates this second phase may be expected to last 2–3 months. The duration of the first phase seems to be influenced by the initial size of comb, but so far as our present results go, lasts from 4–6 weeks. Both curves suggest that there is a temporary slowing down in the rate of shrinkage towards the end of the second week, but in the absence of further information one cannot be sure of this, nor can one offer any explanation. The first and rapid phase of regression probably corresponds to the time when the œdematous layer is being re-absorbed, and the slow second phase to the gradual atrophy of the fibrous and vascular elements of the comb. There is one point of especial significance in connection with this comb atrophy, *i.e.*, the rate of atrophy compared to that observed after adult castration. We are unaware of any records of comb atrophy after adult castration, but an experiment of our own may be used for comparison. An adult male Barred Rock with a comb $8\cdot5 \times 4\cdot3$ cm (area $36\cdot6$ sq cm) rather smaller than that of HF 36, was castrated. Nineteen days later the area had fallen to $22\cdot7$ sq cm, and at 32 days to $17\cdot1$ sq cm, representing 62% and 47% respectively of the original area. At 42 days the area had decreased to 38%. On this basis, the castrate's comb decreased slightly less rapidly than that of HF 36, but more rapidly than that of HF 40. The capon has not yet been killed, but the size reached by the comb after 6 months ($4\cdot8 \times 1\cdot9$ cm) makes it reasonably certain that castration was complete. The rate of atrophy of the fowl comb after hypophysectomy is thus of the same order as after castration; a similar relation is shown on pp. 233-239 for the comb of the Sebright Bantam cock. It seems evident, therefore, that not only is the testis internal secretion decreased immediately after hypophysectomy (as could be deduced from the fact that the comb begins to shrink immediately), but that, judged by its effects, it stops abruptly. So far as we know this point has not been demonstrated on the accessory glands of mammals.

It will be seen from fig. 2 that a small but measurable shrinkage of the comb takes place in the first 3–5 days after hypophysectomy. This was also observed in HF 37 and 42, but not in those receiving a course of anterior lobe extract injections after the operation. At about one week after operation, obvious changes such as blanching and shrinkage of the papillæ are visible. Obvious blanching was observed as early as 5 days and in another case not until 10 days after operation. During the third week the comb and wattles present a remarkable appearance. They are still fairly large but their colour has turned to a washed-out brownish-pink, and the surface is smooth owing to the shrinkage of the papillæ. Subsequently the comb and wattles become dry and parchment-like

and the well-known scaly appearance of the capon comb is seen. In HF 40 the comb at the time of greatest shrinkage oozed quantities of resin-like substance which dried on the outside and then chipped off in lumps. This process, which lasted about 2 weeks, stopped when the

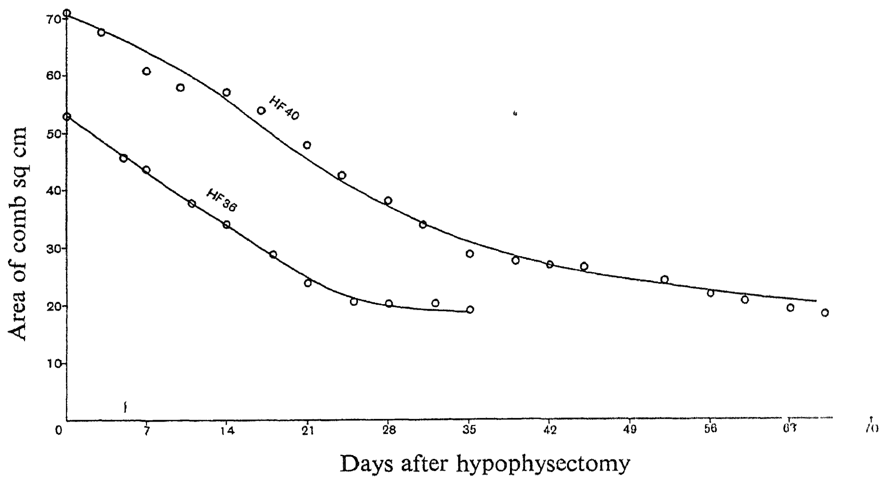

FIG. 2—Atrophy of cock's comb after hypophysectomy

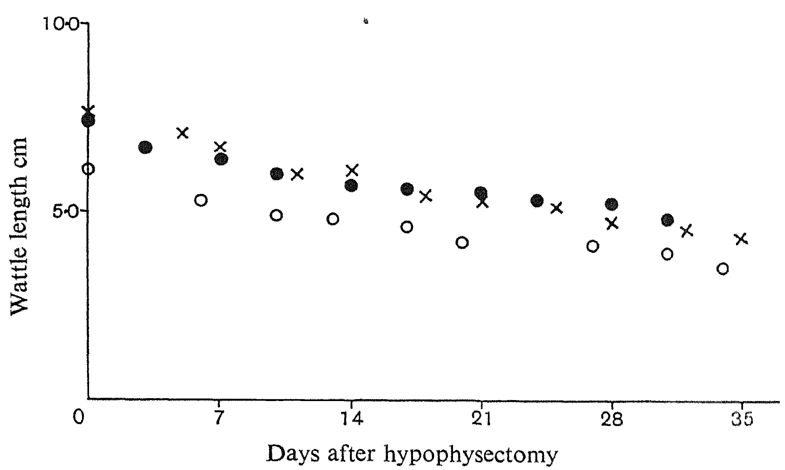

FIG. 3—Atrophy of cock's wattles after hypophysectomy

comb shrinkage began to slacken. Figs. 6–9, Plate 5, give an impression of the atrophy of the comb.

Our histological material of the atrophic comb is limited to one specimen, that of HF 37. The normal adult male comb, as described by Hardesty (1931) consists of a central core of connective tissue and blood vessels, an intermediate layer in which a connective tissue matrix is dis-

tended by mucoid, and a compact peripheral zone rich in capillaries and covered by a stratified epidermis from which keratinized layers are constantly being shed, fig. 10, Plate 6. Castration prevents the mucoid from forming or causes it to disappear, and this produces the smaller paler comb of the capon. The atrophy of the comb after hypophysectomy, as after castration, is brought about essentially by the reabsorption of the mucoid layer. In HF 42, 15 days after operation, in which the comb was only one-half to one-third down, there was a fair amount of mucoid left, but in HF 37 the intermediate layer appears to be represented merely by the depleted connective tissue matrix. The peripheral zone is noticeably less vascular, while the epidermis is thinner, and the desquamated material more abundant, fig. 11, Plate 6.

4—Effect on the Female Fowl

The Ovary—Three of the hens listed in Table II were known to be in full lay at the time of operation. The others were judged to be similar by their comb condition and general appearance. In addition, three other birds which died, 4, 4, and 9 days after operation, were known to be laying. At least six hens, therefore, and almost certainly more, were laying at the time of operation. Some of these laid eggs on the day following operation, but in no case were normal eggs produced later than this. One shell-less egg was produced 2 days after operation. Eggs laid the following day would be in the oviduct at the time of operation, so we may conclude that hypophysectomy stops ovulation rapidly. Since no yolk larger than 6 mm diameter was found 15 days or later after operation, it would seem that the large yolks, which must have been present in some of these hens, were reabsorbed. Several abnormal-looking small yolks were found at autopsy. In the longest standing hypophysectomized bird, no yolks larger than 1–2 mm were found, except one abnormal one about 3 mm in diameter. These results as regards the development of yolks are reflected in the total ovarian weights shown in Table II. It is evident, therefore, that in the fowl, removal of the pituitary not only stops ovulation, but also inhibits the development of the ovarian egg. This fact is of interest, because in mammals full-sized eggs, surrounded by one or two layers of follicular epithelium, are found many months after hypophysectomy.

The ovary of the hypophysectomized hen is essentially similar to that found in the non-laying bird at the height of the moult.

The Oviduct—The oviduct of the laying hen through the region of the albumen gland has a diameter of about 1 cm ; in section the large folds

TABLE II—Hypophysectomized female fowl

Temporary treatment after hypophysectomy	Time of last observation* after hypophysectomy (days)	Ovary		Comb				Wattle length		Notes
				At operation		At last observation*				
		Weight (gm)	Diameter largest yolk (cm)	Length (cm)	Height (cm)	Length (cm)	Height (cm)	At operation (cm)	At last observation* (cm)	
None	44	0·9	0·3	7·7	4·0	5·8	2·3	3·8	3·0	Received cortin during the last 2 weeks.
Cortin	15	2·3	0·5	6·9	4·2	6·3	3·4	4·4	3·6	—
A.P. extract	60 (still living)	—	—	7·1	3·1	5·6	2·5	2·8	2·6	—
,,	51 (still living)	—	—	5·9	3·3	5·1	2·5	2·6	2·5	—
,,	16	4·3	0·4	6·5	2·9	6·6	3·0	3·3	2·8	—
,,	37 (still living)	—	—	6·7	4·2	5·3	2·4	2·9	2·6	A.P. extract injections during 5th week.
,,	21	2·6	0·6	6·9	3·9	5·5	3·0	3·5	2·9	—

* "Last observation" refers to time of death, killing or latest observation on those still living (June 29, 1934).

of the glandular wall almost obliterate the lumen and the tubular albumen glands are very large and distended, while the connective tissue cores of the folds are inconspicuous, fig. 21, Plate 8. The epithelium lining the lumen is high columnar and ciliated. In HF 64, known to be laying at the time of operation, the oviduct was 22 cm long 16 days later at death. The diameter at the albumen gland was about 0·4 cm and in section the individual glands were atrophic and contracted, and contained no albumen. As a result the folds were small and the lumen relatively large. The epithelial layer had sunk to low columnar and cilia were scarce. A similar condition was seen in HF 51, fig. 22, Plate 8, and in HF 66, the former of which was known to be laying at the time of operation. At 44 days after hypophysectomy the process has gone further. The whole tract is much smaller and the glands, epithelium, and folds even more atrophic, fig. 23, Plate 8. As with the ovary, the condition of the oviduct found after hypophysectomy is typical of that found in the non-laying hen.

Comb and Wattles—The comb of the laying hen is similar in essentials to that of the cock. There is a well-developed layer distended with mucoid. In the non-laying hen this intermediate layer disappears and the comb shrinks and collapses. These changes are associated with the plump red comb of the laying hen turning to the shrunken brownish one of the non-laying bird. After hypophysectomy of the laying hen the mucoid also disappears and a comb typical of the non-laying hen is produced. As with the cock's comb, however, the ultimate degree of atrophy, as determined by size, appearance, and feel, is greater than is found in the inactive adult. Similar changes are observed in the wattles.

The atrophy curves of the hens' combs are complicated by the fact that all except two of our hens received temporary replacement therapy after operation. This in most cases caused a temporary increase in the size of the comb, and HF 64 had barely returned to normal before death at 16 days after operation. The others, however, all showed a net decrease at the final observation. The curves for the two birds not receiving anterior lobe injections after operation are shown in fig. 4. The others will be dealt with in a later paper in considering the effect of replacement therapy on the comb size.

5—Observations on other Birds

Turkey—Hypophysectomy of an adult male turkey was followed by fading of the brilliant colours of the skin on the underside of the head and by atrophy of the top-knot. The bird was killed 3 weeks after

operation. The testes weighed 3·9 gm. We have no information as to the normal size of the testes in turkeys, but by analogy with fowl, they may well reach 100 gm. Histologically they showed almost the same condition as those of HF 42, but atrophy was rather further advanced fig. 18, Plate 7. The vasa deferentia were also clearly atrophic, though they still contained spermatozoa showing that the bird had previously been fertile.

Sebright Bantam—The chief interest of the Sebright bantam, of course, lies in its abnormal plumage, but the record of a Sebright cockerel which died 39 days after hypophysectomy may suitably be included here. This bird was given anterior lobe extract injections for 4 days after operation

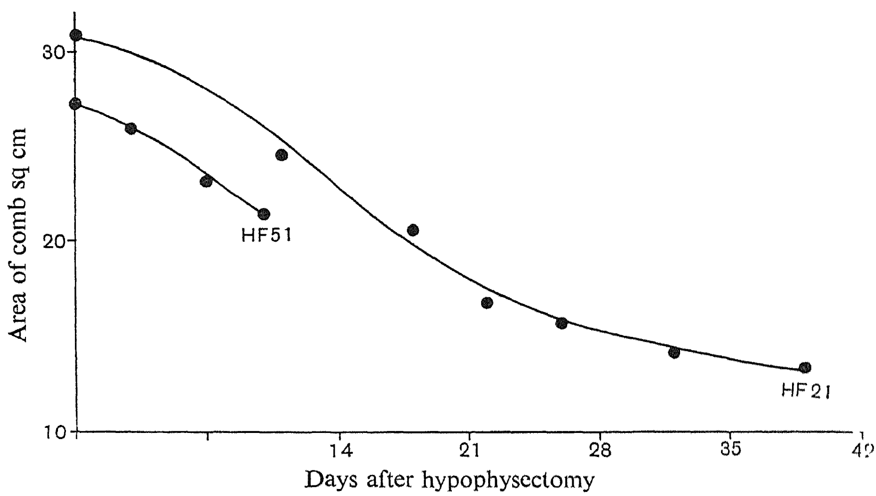

FIG. 4—Atrophy of hen's comb after hypophysectomy

and maintained good condition, losing only 80 gm in body weight during the 39 days. At about 2 weeks after operation a heavy moult started, and within 10 days the bird was practically bare except for a few primary wing feathers. Death coincided with a failure of the arrangements made to keep the bird warm and was undoubtedly due to loss of body heat. There was no evidence of a slow decline in condition in this bird.

The testis were obviously atrophic, weighing only 0·255 gm. The testes of four normal Sebright cocks weighed 4·2, 5·9, 6·1, and 8·5 gm, so that those of the hypophysectomized bird had fallen to about 5% of the normal weight. This proportionate decrease in testis weight is very similar to that observed in HF 37.

The testes of the Sebright were histologically similar to those of HF 37 and need not be described in detail, fig. 19, Plate 7. The

tubules measured about 45 μ in diameter as against about 230 μ for the normal Sebright testis tubule. The vas deferens was similar to that of HF 37.

The comb of this bird, which was large and a brilliant scarlet in colour at the time of operation, regressed very rapidly. Fig. 5 shows the decrease in the comb length × breadth index (a rose comb) after hypophysectomy. The decrease in size appears to be more rapid than in the case of the Leghorns; this may be due to the initial size of the comb or to the difference in type. If the rose comb atrophies more quickly than the single type, the response to testis hormone might be more rapid, in which case rose comb capons might be valuable for assay work.

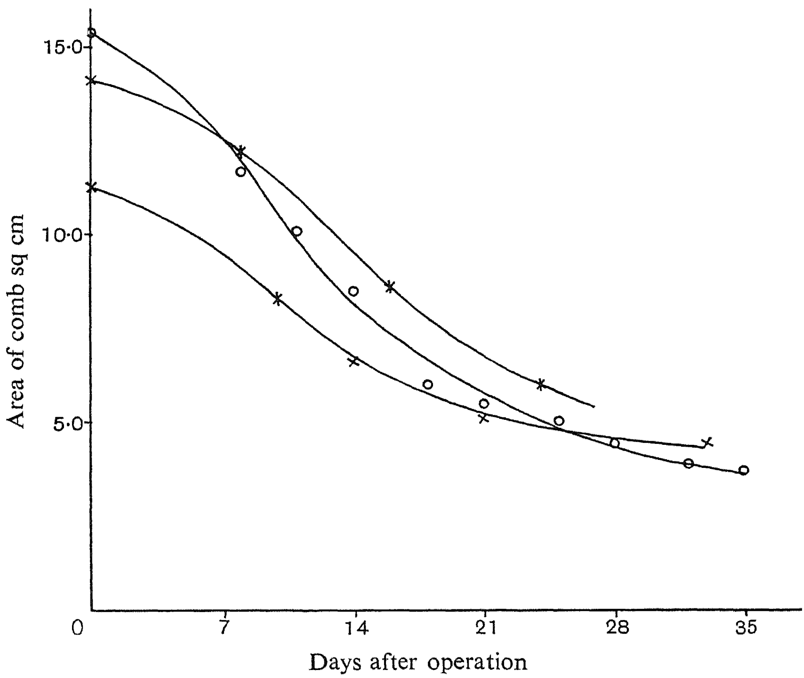

FIG. 5—Comb of Sebright bantam, × after castration, and o after hypophysectomy

We are able to compare the comb atrophy in the hypophysectomized Sebright with that found after castration in two similar bantams. The curves shown in fig. 5 are substantially similar, and thus confirm the result on Leghorns, that removal of the pituitary causes a rapid cessation of internal secretion by the testis.

Histologically, the comb of the hypophysectomized Sebright showed

the resorption of the mucoid layer and general decrease in vascularity typically observed after castration, figs. 12 and 13, Plate 6.

Pigeon—Two hypophysectomized male pigeons were available. Laparotomies were carried out on both before the hypophysectomy to ascertain whether the testes were fully developed. The first, in which the testes were only about one-half full size at the time of operation, died 13 days later. The testes were minute, weighing only 0·09 gm. The tubule diameter was 60 μ. Histologically, the tubules contained little more than spermatogonia and a few spermatocytes, and the intertubular tissue was relatively abundant. The second pigeon had fully developed testes at the time of operation and died 12 days later. The testes at death were very atrophic; they weighed 0·19 gm, and had a tubule diameter of 90 μ. Histologically they were similar to HF 42, and contained no stages later than spermatocytes, though the intertubular tissue was not yet relatively greater in amount, fig. 17, Plate 7. The less atrophic condition of these testes as compared with those of the first pigeon is clearly due to the latter not having been fully developed at the time of operation.

The normal active pigeon testes weigh 1·5–3·0 gm and the tubule size is 180–200 μ (Marrian and Parkes (1928)).

6—Discussion

The observations recorded above make it evident that, as in mammals, the proper functioning of the gonads and thence of the accessory reproductive organs of fowls is dependent on the presence of the pituitary body. We have further extended this generalization to include a secondary sexual character which is dependent on the gonad, *i.e.*, the comb and wattles. The evidence that the anterior lobe is the organ involved is not yet complete, but this may be confidently assumed.

Since the atrophy of the cock's comb after hypophysectomy takes place at the same rate as after castration, it may be assumed that the fowl testis is extremely sensitive to pituitary insufficiency, and that secretion of the testis hormone stops as soon as the pituitary is removed.

The changes which follow hypophysectomy may be compared with those in normal birds at the end of the laying season when the moult sets in. The former changes, as described in this and the preceding paper (Hill and Parkes (1934)) include regression of the gonads and reproductive tract, and of the comb and wattles, a temporary loss of body weight, and complete moulting. The normal cock when it begins to

moult shows a loss of body weight, decrease in the size of the comb and loss of fertility. In the moulting hen the parallel is complete, including stoppage of laying, regression of ovary and comb, and loss of body weight. The difference seems to be only one of degree, the changes after hypophysectomy being much more severe than in the moulting normal bird. This, however, would be expected, and the evidence warrants the conclusion that the occurrence in the normal bird of a phase characterized by moulting, etc., is conditioned by a temporary, partial pituitary deficiency. To this extent it is obviously comparable with the anœstrous period of mammals.

We are indebted to Dr. A. W. Greenwood, Institute of Animal Genetics, Edinburgh, with whom we have been able to discuss the results from time to time.

7—Summary

Hypophysectomy causes rapid atrophy of the testes of the adult cock. The tubules become devoid of spermatozoa in two weeks, and by five weeks contain nothing but spermatogonia, and a few Sertoli cells. There is a marked relative increase in the amount of intertubular tissue. The comb and wattles regress in much the same way as after castration.

Similar results have been obtained in a turkey, a Sebright bantam and a pigeon.

Hypophysectomy of the laying hen results in the cessation of ovulation, and the ovary, oviduct, and comb regress to the type characteristic of the non-laying bird.

It seems highly probable that the changes associated with the moulting season in fowl, loss of fertility, comb regression, loss of body weight, etc., are due to a temporary pituitary deficiency.

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Description of Plates

Plate 5

Fig. 6—HF 40, 10 days after hypophysectomy, atrophy of comb and wattles just begun.

Fig. 7—HF 40, 63 days after hypophysectomy. Same magnification as fig. 1, showing comb and wattle atrophy, and growth of new feathers.

FIG. 8—HF 40, 63 days after hypophysectomy, showing general condition.

FIG. 9—HF 36, 35 days after hypophysectomy. At this time the comb had decreased to one-third its original area.

PLATE 6

FIG. 10—Comb of normal Brown Leghorn cock, showing peripheral layer, distended mucoid zone and central core. × 15.

FIG. 11—Comb of HF 37, 35 days after hypophysectomy, showing increased sloughing of the epidermis, decrease in the peripheral layer, and disappearance of the mucoid zone. × 15.

FIG. 12—Cross-section of whole rose comb of normal Sebright bantam, showing great development of the mucoid zone. × 6.

FIG. 13—Cross-section of whole rose comb of Sebright bantam 39 days after hypophysectomy, showing atrophic changes similar to those in HF 37. $8\frac{1}{2}$.

PLATE 7

(All × 200)

FIG. 14—Normal cock testis, for comparison with following figures.

FIG. 15—Testis of HF 42, 14 days after hypophysectomy.

FIG. 16—Testis of HF 37, 35 days after hypophysectomy, showing profound atrophy of tubules and relative increase in the intertubular tissue.

FIG. 17—Testis of pigeon, 12 days after hypophysectomy.

FIG. 18—Testis of turkey, 21 days after hypophysectomy.

FIG. 19—Testis of Sebright bantam, 39 days after hypophysectomy ; condition similar to that of HF 37.

PLATE 8

FIG. 20—Testis of HF 37, 35 days after hypophysectomy, showing general topography, and thickening of the tunica. × 75.

FIG. 21—Folds of the albumen gland of oviduct of laying hen, for comparison with figs. 22 and 23. × 16.

FIG. 22—Oviduct of HF 51, at the albumen gland, 15 days after hypophysectomy. Same magnification as fig. 21.

FIG. 23—Oviduct of HF 21, 44 days after hypophysectomy, showing complete atrophy of the albumen gland. Same magnification as fig. 21.

FIG. 24—Ureter and vas deferens of normal cock. × 24.

FIG. 25—Ureter and vas deferens of HF 37, 35 days after hypophysectomy, showing complete atrophy of vas. Same magnification as fig. 24.

FIG 6

FIG 7

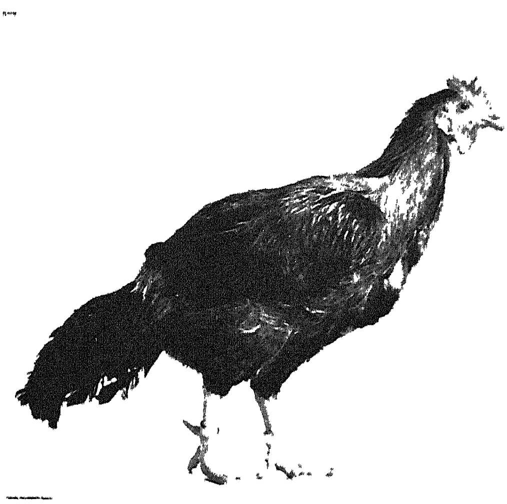


FIG. 8

FIG. 9

(*Facing p.* 236)

FIG. 10

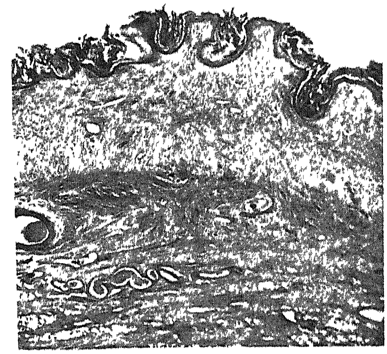
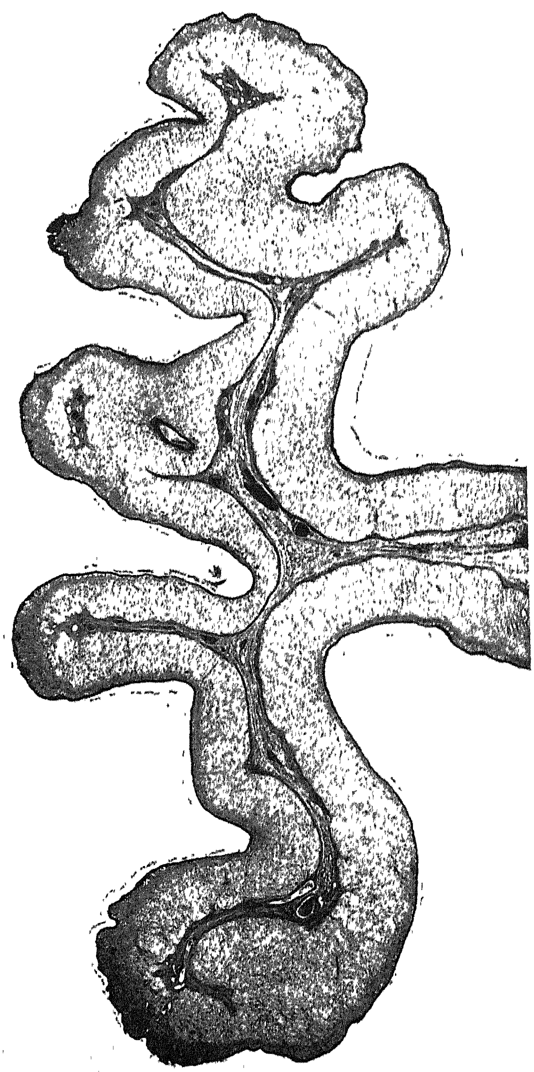


FIG. 11

FIG. 13



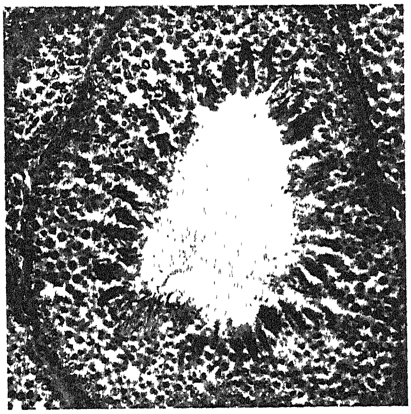


Fig. 14

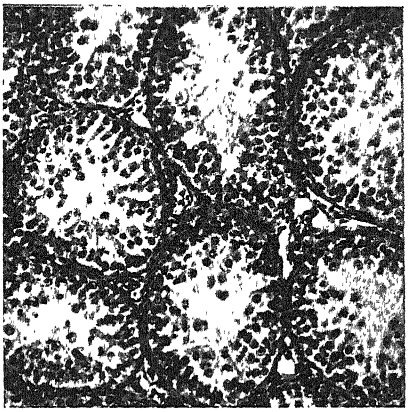


Fig. 15

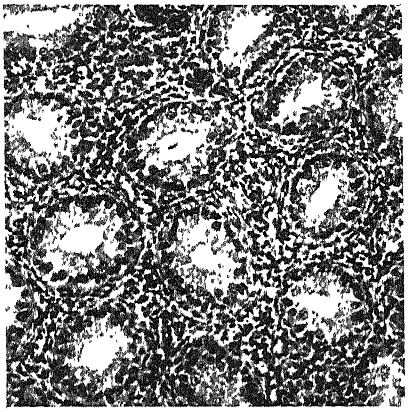


Fig. 16

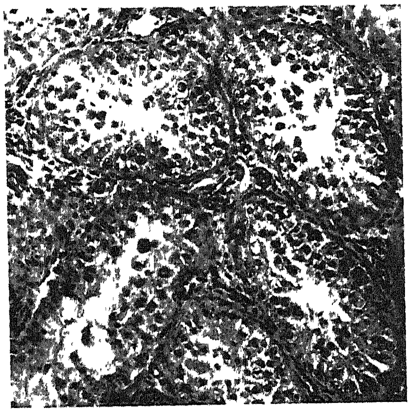


Fig. 17

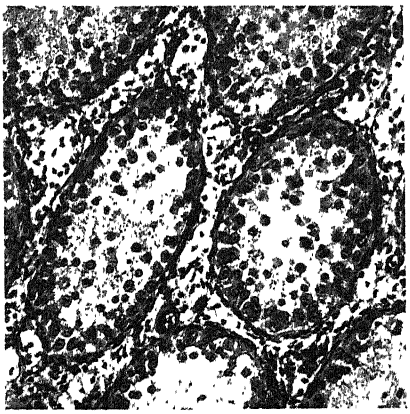


Fig. 18

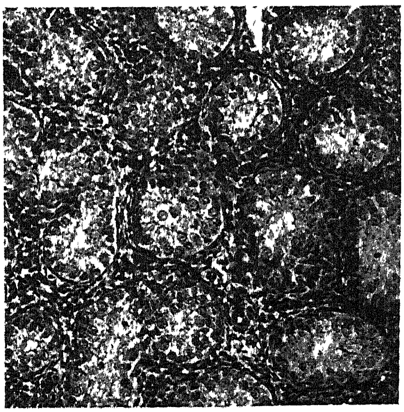


Fig. 19

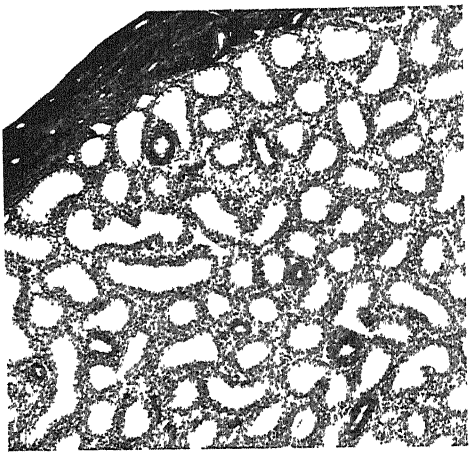


Fig 20

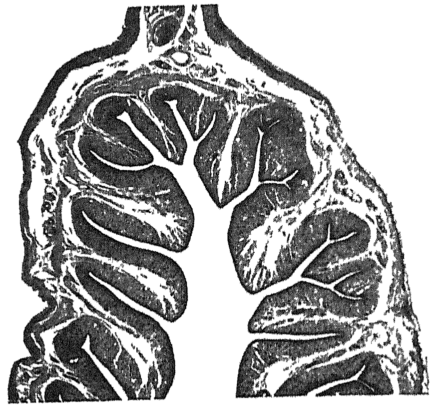


Fig 22

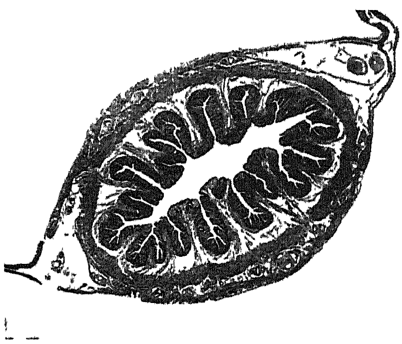


Fig 23

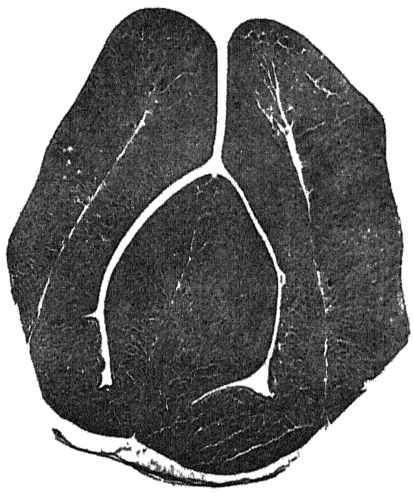


Fig 21

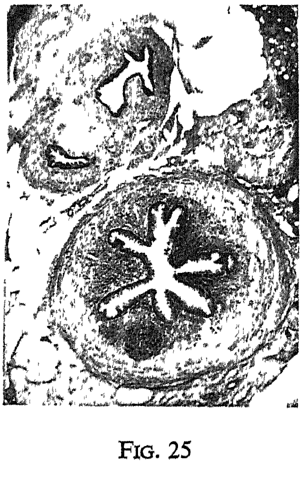


Fig. 25

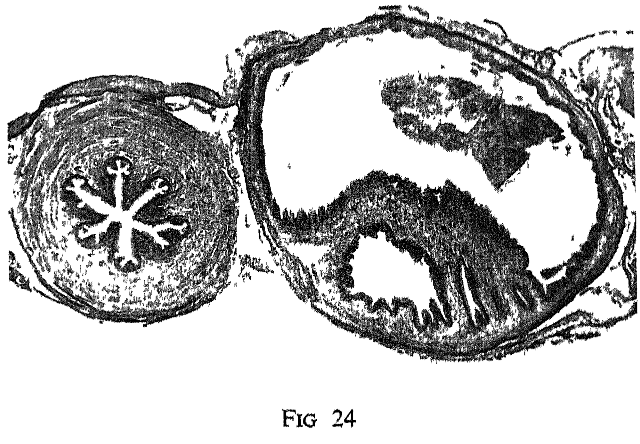


Fig 24

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Biological Methods of Diagnosing Equine Pregnancy
I—The Mouse Test

By W. C. MILLER, Institute of Genetics, University of Edinburgh

(*Communicated by D. M. S. Watson, F.R.S.—Received April* 4, 1934)

INTRODUCTION

In the larger domesticated animals, clinical methods for diagnosing pregnancy are inadequate or unsatisfactory during the first third of the gestation period, and in individual instances for a longer period. In comparison with most other animals, œstrus in horses is markedly erratic, for a pregnant mare may continue to show from one to three apparently normal œstral manifestations after conception, and may even accept service on each occasion. On the other hand, unsuccessful coition, in a considerable proportion of cases, may inhibit a subsequent œstrus. In addition, particularly in highly-bred thoroughbreds, only one œstrus period may, in certain circumstances, be exhibited during a breeding season, and the non-appearance of subsequent heat cannot be regarded as evidence of conception. Neither the clinician nor the owner, therefore, can obtain reliable evidence of the existence of pregnancy from observation of the sexual behaviour of the mare.

Clinical methods, necessitating examination of uterus or ovaries by rectal and vaginal methods, are cumbersome, difficult, sometimes dangerous or impossible, owing to the temperament of the mare, and except to the thoroughly experienced veterinarian these methods do not always yield reliable evidence of pregnancy until from 3/11 to 5/11 of the gestation period have passed. Numerous instances have been encountered where a valuable thoroughbred mare or hunter has been prevented from working in autumn, and during winter has been treated mistakenly as though she were pregnant, sometimes at considerable inconvenience and expense; similarly other mares not infrequently exhibit markedly subnormal subjective signs of pregnancy and may continue to be regarded as barren until a very short time (sometimes only two weeks) before parturition.

It is evident, therefore, that a reliable method of diagnosing early pregnancy in equines has important practical applications and may be instrumental in saving expense and inconvenience to the owner and hardship or cruelty to the mare.

The biological test for pregnancy to be described is based on the presence of œstrin in the urine of the gravid mare. The original investigations relating to the physiological effects of sex hormones and their identification in urine during pregnancy carried out by numerous workers, in particular by Evans and Long (1921), Allen and Doisy (1923), Zondek and Ascheim (1927), and Kahnt and Doisy (1928), are sufficiently well known. Zondek and Ascheim made use of the presence of anterior pituitary gonadotropic hormone in urine to diagnose pregnancy in the human female. Prepared urine was injected into immature female mice and upon the presence or absence of hæmorrhagic or atresic follicles and corpora lutea in the ovaries of these, a positive or negative diagnosis was based.

The urine of the mare has been investigated by several workers in relation to the presence or absence of gonadotropic anterior pituitary hormone (prolan), and there is now a definite consensus of opinion that no readily recognizable quantities of this hormone are excreted during pregnancy; these findings have been verified by the present writer. Cole and Hart (1930, *b*), Zondek (1930), and others have shown, however, that blood serum of pregnant mares from about the 42nd day of pregnancy onwards until about the 80th to the 100th day (after which diminishing reactions were obtained), contained recognizable quantities of gonadotropic hormone, and that its identification during this period might be utilized as a basis for diagnosing pregnancy. Shtamler, Shukhgalter, and Faiermark (1933) prefer to use the prolan content of the blood serum on which to base a diagnosis of early pregnancy, but they report a high incidence of error in a comparatively few cases (4 errors in 143 tests).

The work to be reported here has been entirely based upon the presence of the œstrus-producing or cornifying hormone (œstrin or folliculin) in the urine of the mare. It is unnecessary to review in detail the large body of evidence which has now been accumulated to confirm the findings of Zondek, Küst (1931), Cole and Hart (1930, *a*), Anderson (1934), Crew, Miller, and Anderson (1931), and others, that equine urine contains gradually increasing quantities of œstrin from about the 60th day onwards. The fact is so well established that equine urine is now being used as a recognized commercial source for the production of œstrin for physiological and therapeutical purposes.

Work on the diagnosis of pregnancy in mares by means of injection of prepared urine into oöphorectomized female mice commenced at this Institute in 1930. For much of the preliminary work carried out by Crew, Miller and Anderson (1931) Shetland pony mares were used. From

experience with these it was possible to determine convenient methods of securing the sample, its preparation, and the relationships between duration of pregnancy, age of sample, and the results obtained. The following account regarding the methods adopted at present is the outcome of that work, reinforced by a much wider experience of testing samples sent by owners subsequently.

Age of the Urine Sample

A test for pregnancy can obviously be of greatest utility if it can be performed during the early stages of pregnancy. The samples received

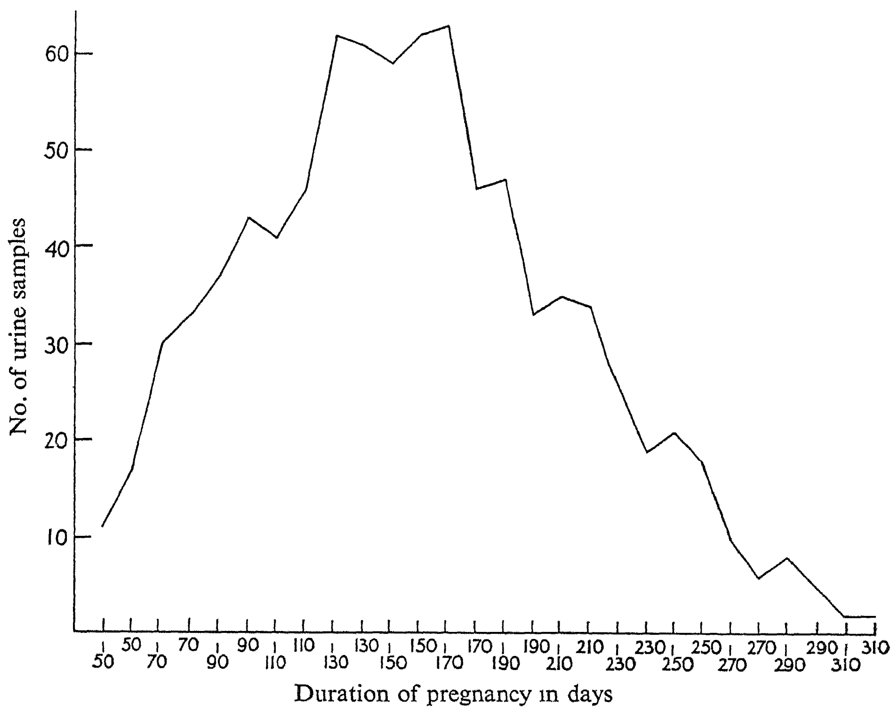

Fig. 1

during the initial stages of the investigation represented nearly the whole of the gestation period, but more recently the greater proportion of samples sent in have been secured about the 170th day of the gestation period as fig. 1 indicates.

Since œstrin in a sample of urine appears to be thermo-stable, and since the only adverse change would appear to be that caused by excessive oxidation from exposure to the atmosphere, it was found that no special

measures were required to keep samples at a low temperature or to carry out the test within a given time after collection. The only precaution found necessary has been with samples from India. Owing to the elapse of some 23 to 27 days before receipt, bacterial decomposition (ammoniacal) was excessive, and to prevent oxidation and inhibit bacterial action a seal of toluol was employed over each sample.* This proved markedly effective in that unsealed samples, even after treatment, proved toxic to a proportion of the mice used while those which had had about 10 cc of tuluol added to each 100 cc of urine showed no toxic effect when injected. Samples from Kenya, which are now sent by Air Mail, arrive 7 to 8 days after collection and these are the oldest ones dealt with at present. Continental samples and those from Ireland may be 3 to 5 days old on arrival, but it is rare for other samples to be more than 48 hours old when they are received here. In none of these is it necessary or advisable to add either preservative or seal.

Age and Treatment of Mice

The mice used are generally obtained when about 3 to 4 weeks old and are oöphorectomized immediately. They may be used 14 days after operation, but it is better to allow at least 3 weeks to elapse before the first injections are given. After the completion of a test, an interval of at least 10 days should elapse before the same batch of mice is used again.

It has been noted that as the mice approach the age of 15 to 18 months the cornifying response to injections of urine with a fairly high œstrin content becomes slower. To avoid the chance that such old mice might fail to respond to urine with a low œstrin content from a mare in the early stages of pregnancy only young mice are used for samples taken less than 60 days from the date of service. Mice are dispensed with when about 20 months old, or if they become too fat sooner than this.

Preparation and Infection of the Sample

Owing to the high percentage of mucin characteristic of equine urine, and because of the bacterial content of samples collected in stables where every opportunity for contamination of the sample exists, it was found

* Samples of urine from female elephants—some 27 tests of which have been made without any reaction either for gonadotropic anterior pituitary hormone or œstrin—were much worse than equine samples in this respect, but a toluol seal enabled these samples to arrive in a much better condition.

necessary to prepare the urine before injection. Samples which were not previously subjected to any form of preparation were found to be highly toxic for mice, death occurring in from 24 to 96 hours after first being injected (varying to some extent with individual samples).

Originally, one gram of sulpho-salicylic acid per 25 cc of whole urine was used to precipitate the mucin and the bulk of the proteins. Thereafter, the sample was neutralized by adding sodium bicarbonate to give a p_H of about 7·0 to 7·5 (B.D.H. Universal Indicator was used). After this treatment was adopted there were still about 12% of instances encountered in which lethal effects followed injection. Equine urine varies in p_H from highly alkaline to highly acid ; the former condition generally obtains when the animals are at grass. The addition of 1 gram of sulpho-salicylic acid per 25 cc of urine is sufficient to neutralize specimens exhibiting the higher degrees of alkalinity and yet leave in the sample an amount of free sulpho-salicylic acid sufficient to exert full inhibiting effects on organisms and sufficient to precipitate proteins. The method now adopted with highly alkaline urine is to add the acid until all reaction between it and the sample ceases; to add in addition at least 1 gram for every 50 cc ; then to agitate thoroughly and allow to stand for half an hour. This gives an excess of the acid sufficient to effect the necessary degree of detoxication and precipitation. Thereafter the urine is neutralized and filtered to remove precipitates and any contaminating debris, sand, particles of dung, etc. The system adopted has given very satisfactory results and at present such deaths as do occur are rare and occur only with samples from diseased mares. Septic conditions of the equine uterus will always be a potential cause of the death of test mice, owing to gross contamination of the sample with streptococci, but a habit is made of requesting owners, when forwarding samples, to indicate any abnormal condition in the mares.

It has been the custom to divide mares into three classes according to the duration of pregnancy when the sample was collected, as follows :—

Class I—from 40 to 60 days;
Class II—from 60 to 80 days;
Class III—from 80 to the end of the gestation period.

With samples of the first class, two oöphorectomized mice are injected with normal urine, two with urine diluted to twice its volume with distilled water, and two with urine concentrated by evaporation by boiling to two-thirds of its original volume.

The amounts given ensure that a range between 0·6 and 2·4 cc of whole urine are received by the various mice as in Table I.

TABLE I

Urine	Amount given twice daily	Total actual urine received
N/1	0·2 cc (× 6)	1·2 cc
N/1	0·4 cc (× 6)	2·4 cc
N/0·5	0·2 cc (× 6)	0·6 cc
N/0·5	0·3 cc (× 6)	0·9 cc
Conc. 2/3	0·2 cc (× 6)	1·8 cc
Conc. 2/3	0·25 cc (× 6)	2·2 cc

The dosages in Table I refer to the amounts of an average sample from a mare from 50 to 60 days pregnant. For earlier stages than this each injection is increased by 0·05 cc, giving a range of 0·75 cc to 2·7 cc of actual urine. For a positive diagnosis the three mice receiving the maximum doses must show complete cornification, and at least two of the others must yield a picture in which there is a definite majority of cells in early incomplete cornification. The details of these smear pictures will be explained later.

For mares, in what for convenience is designated Class II of pregnancy, the dosages used by Anderson (1934) are usually adopted, unless from the history supplied there is reason to believe that excretion of œstrin may be slower than normal, or that the concentration may be low. In these instances the mare is dealt with as though she belonged to the Class I. Instances of this nature are mares which (i) are aged and have not been bred from with regularity in previous years, especially those of over 18 years; (ii) are primiparous; (iii) have been barren during the previous breeding season; or from experience of them in previous years are known to be slower than normal in mobilizing their œstrin-producing mechanism.

Otherwise, the dosages are as shown in Table II.

TABLE II

Urine	Amount given twice daily	Total actual urine
N/1	0·3 cc (× 6)	1·8 cc
N/0·3	0·35 cc (× 6)	0·7 cc
N/0·3	0·4 cc (× 6)	0·8 cc
N/0·3	0·5 cc (× 6)	1·0 cc
N/0·3	0·6 cc (× 6)	1·2 cc

For a positive reaction at least three mice including that receiving normal urine must show full cornification. In samples containing the normal amount of œstrin for this class of mare, all the mice, or all except that receiving the minimal dosage, usually show a fully cornified smear.

For mares which are over 90 days pregnant, the above dosages are reduced by 0·1 cc in each case and only the first four mice are generally used. It is customary to obtain full cornification from each mouse in the great majority of positive tests, and seldom is there more than one which is not at least partially cornified, unless by accident, a mouse has been used during the refractory stage.

Smearing and Staining

After injections have ceased, a period of 36 hours is allowed to elapse and a smear of the vaginal contents is taken from each mouse. The smears are stained by the Giemsa technique.

Results Obtained

The reactions in the vaginal epithelium of the test mice are those which normally characterize œstrus in this animal. In consequence of the work of Allen and Doisey, Long and Evans, and others, the typical appearances of a fully cornified vaginal smear are well known. Very recently cornified cells stain a brilliant blue by Giemsa, usually show the ghost-like remains of the nucleus, placed centrally, and are free from the mosaic pattern which characterizes other older cells. These latter stain a bluish pink, or are actually reddish in some cases. They present the same outlines, but in addition are usually characterized by a peculiar mosiac pattern, which is due to erosion of the surface or the substance of the cell by keratinophagic organisms of a streptococcal nature. These stain readily and are easily seen. Full cornification always indicates pregnancy in the mares concerned, when the doses referred to earlier are used.

Negative smears are those characteristic of the normal diœstrum.

In doubtful reactions, which must always be considered in relation to the age of the mares and the duration of pregnancy, the smear is intermediate between a normal positive and a normal negative one. There may be 40% to 60% of the cells present cornified, with a preponderance among them of cells showing traces of nuclei. These appearances are usually characteristic of samples which contain only a small amount of œstrin and the reaction is proportional to the amount of injection each mouse receives.

Since it is known that the excretion of œstrin increases rapidly during pregnancy a reaction which would constitute a positive result at, say, 70 days would be regarded as a doubtful at 160 days. Generally speaking, it has been found that doubtful reactions as defined above are obtained

from mares (up to about the 70th day of pregnancy) which fall into one of the following categories : (i) young primiparous pregnant mares ; (ii) those which were barren the preceding year or for two years, but are pregnant at the time of testing ; (iii) those which are not pregnant, but which are suffering from endometritis or possibly have luteal cysts in their ovaries. The evidence on the latter point is not yet conclusive, but from the subsequent history of two such mares, the genitalia of which were eventually examined *post mortem*, and from a consideration of the general behaviour (evidence of intractability and incipient nymphomania) of two others it seems permissible to suggest ovarian dysfunction due to cysts as a possible cause of the excretion of larger amounts of œstrin than the normal for a barren mare.

Doubtful reactions given by mares which, had they conceived, would be between the 140 and 200 day of the gestation period are rare and appear to be associated in a number of cases at least with clinically demonstrable chronic endometritis or leucorrhœa, possibly consequent upon infection at a previous parturition. Doubtful reactions, where more than 230 or 250 days have elapsed since the last service, when not falling into one of the classes already discussed, may have been from mares which, unknown to the owner, were in œstrus when the sample was collected.

One other possibility of error must be mentioned. Either through imperfect technique during oöphorectomy or possibly owing to regeneration following the operation, one mouse in a batch may occasionally yield a cornified smear, the others giving diœstrous smears. At present, tests in which a result of this nature occurs are considered to be negative, the single mouse giving cornification being killed. Ovarian tissue has been histologically recognizable in two such instances, while in the others an ovary-like body was present on one side, but an absolute diagnosis was not made.

Practical Applications and Accuracy

The total number of tests which have been carried out up to the present is 1303, but this figure includes some 320 preliminary tests, which must be regarded as experimental and necessary to determine standards. Of the remaining 983 tests, 543 gave positive reactions and 412 gave negative results, while 28 were doubtful. It has been the custom whenever a doubtful reaction was obtained to secure a second sample either almost immediately, or, if it appeared probable that the mare was in season when the first sample was collected, at such time as would ensure that the

collection of the second sample did not coincide with a succeeding period of œstrus. In this way, it has been possible to give a definite diagnosis of the condition of the majority of such mares as gave a first doubtful reaction, but for three mares a third sample was required. Probable explanations of doubtful reactions which are only offered tentatively at present are that :—

(i) The excretion of œstrin from a normal non-pregnant mare was temporarily in excess of the usual quantity, possibly due to feeding, exercise, or excitement just prior to the collection of the sample.

(ii) Excretion of œstrin was abnormally high owing to irritation or inflammatory change in the ovaries of a non-pregnant mare. Some preliminary evidence has been collected to show that mares which possess ovarian cysts and those which exhibit nymphomania excrete a considerable amount of œstrin in their urine. Similarly, mares suffering from chronic endometritis (diagnosed clinically afterwards) may excrete sufficient to provoke a weak positive reaction in the test mice. Such mares are probably unable to breed until the infective condition has been overcome by treatment.

(iii) Excretion of an inadequate amount of œstrin during early pregnancy, and possibly also during later stages (although no such instances have yet been encountered).

The circumstances which govern the amount of œstrin excreted, either during pregnancy or when a mare is barren, are not yet fully understood, but it is readily evident that there is a very considerable degree of individual variation. At the present stage of the investigation, little can be done to eliminate the possibility of further doubtful reactions being encountered, but it is possible that with a more comprehensive clinical history available many doubtful reactions could be ascribed to one of the above conditions. Moreover, it is important to note that of the 28 doubtful reactions the majority were obtained from samples from mares between 40 and 70 days after service.

Of the tests completed (*i.e.*, in which confirmatory evidence has been obtained), and including 196 of the later experimental group in which there were four errors, 648 have proved to be correct, five were wrong at the first test, but second samples from these enabled a correct diagnosis to be given. Seven, including the four errors mentioned above, were definitely incorrect ; two were doubtful, and it has been impossible to discover whether these mares were pregnant or not since they were sold by auction to go abroad.

If the four incorrect experimental results are included the error of the test under applied conditions is 1·08%. It is not considered justifiable,

however, to include as errors the five results which were wrong at the first test but right at the second, since none of these gave an initial reaction which could be regarded as definitely positive or negative and the fact that these reactions were indefinite led to a request for second samples, by which a check could be made on the first result.

If the four incorrect results referred to earlier are not included, the error is reduced to less than 0·5%. This percentage is satisfactory when it is remembered that not only is the test sample a biological one subject to a certain unknown degree of fluctuation in its composition and the concentration of œstrin it contains, but the animals used for test are themselves subject to individual variations over which there is no control. It is obviously not possible to give results which include all the mares tested to date, since verification of the diagnosis in many of those recently tested will not be obtainable until nearer the foaling season during the current year. There are 531 mares for which results cannot be verified until later, and in which the error is not, of course, known. There seems no reason, however, to anticipate that it will be greater than in those for which final results have been given above.

SUMMARY

A method of diagnosing pregnancy in mares, based upon the œstrin content of the urine and the reaction produced in oöphorectomized mice, is described. It is shown that under applied conditions the method adopted gives satisfactory results, with an accuracy of at least 99·5%. In a proportion of cases pregnancy can be determined as early as the 42nd day after service, and a definite opinion can be given in the great majority of cases by the 60th day.

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Biological Methods of Diagnosing Equine Pregnancy II—The Capon Test

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(*Communicated by D. M. S. Watson, F.R.S.—Received April* 4, 1934)

[Plates 9 and 10]

Introduction

From a series of experiments on Brown Leghorn fowls Juhn and Gustavson (1930) suggested that the production of the red pigment in the breast feathers of the castrated male of this breed might be used as an indicator of the female hormone (œstrin). They were able to demonstrate the feminizing effect on the breast feathers of the capon within 48 hours of the injection of a single dose of œstrin of the order of 600 rat units. This suggested the possibility of extending the use of the fowl test for œstrin, and of ascertaining whether or not the amount of this hormone in the urine of the pregnant mare might be of such a concentration that by injecting suitable amounts of urine, a diagnosis of pregnancy could be obtained as early as 48 hours after injection. If this could be done the method would be of considerable practical value since the mouse test requires a period of five days from the initial injection before a result can be obtained. With this object a small series of tests have been carried out, and while it will be impossible for some months to verify or disprove the diagnosis from actual facts, they have been controlled and compared as regards results, sensitivity and uniformity with mouse tests on the same sample, the latter, up to the present having given over 99% correct diagnosis.

Description of Plumage

Since the method may prove to be of practical import, the details of the experimental technique will be given at considerable length.

Although the area of the plumage selected for the test has been restricted to the breast region, any of the sexually dimorphic regional feather groups responds characteristically to the hormone stimulus, changing from the male plumage type to the female. The change in the character of the plumage, however, is more easily seen in the breast than in any other region of the body, and, moreover, cannot be confused with the possible effect of a hyperthyroid condition (see later).

Sex dimorphism of the characters in the plumage of the Brown Leghorn fowl relates not only to the colour of the feathers but also to the structure. With the exception of the neck hackle all the feathers of the female are solid and unfringed while in the male such a structure is only found in the breast, the tail and the longer feathers of the wing. It has been shown that the structural features of the feathers can be greatly modified, not only by the administration of œstrin to a male but also by the induction of a hyperthyroid condition. Where the latter is present the tendency is for all feathers to become solid owing to the growth of barbules along that portion of the feather which normally fails to exhibit them and has thus a fringed appearance.

In the female the breast plumage is salmon coloured, usually a pure self colour, but it occasionally shows very tiny and faint black flecks ; the tail and primaries are black or rusty black and the neck hackles pale yellow or straw coloured on their fringed edges. Everywhere else the plumage is of a uniform drab colour, the vane of the feather being of a brownish hue, broken by much fine black pencilling.

The male of this breed possesses a brighter plumage than the female and the number of its distinct colour areas is also greater; the breast, primaries, tail, wing coverts and the wing edge feathers are a solid black; the neck hackle, saddle, back and wing bow feathers are of different shades of red, varying from a bright gold on the neck to a deep mahogany red on the back and wing, the saddle area being of an intermediate shade. The wing secondaries exhibit a reddish edge. Practically all the red feathers show a little black pigmentation near the basal end of their vanes and in the long neck and saddle ones this may extend to form a fine central streak. A type of mismarking is sometimes found in the breast of this sex also and takes the form of red streaks or splashes on the normally black feathers. Birds showing this aberration to any marked extent should be avoided in the assaying of œstrin solutions.

It has been shown by various workers (Goodale (1916); Finlay (1925); and Greenwood and Blyth (1929)) that while the plumage of the normal female of this breed depends for the maintenance of its characteristic pattern and colour on the presence of ovary (secreting ovarian hormone), the plumage in the male is not significantly affected by the presence or absence of testis and is, in fact, of the neutral or asexual type exhibited by the completely gonadless bird of either sex. (The assumption of typical female plumage ensues as a result of the successful implantation of ovary into such individuals.)

Preparation of the Bird for Injection

Since pigmentation, once laid down is not affected by administration of female hormone, growing feathers are necessary for the test. In castrates, which are the type generally used, because there is a continual casting and renewing of feathers always in progress, a number of suitable feathers can usually be found; the fact that they may be scattered all through an area is a disadvantage in that the power of the feathers to react changes in an antero-posterior direction within a region (Lillie and Juhn (1932)) with the result that they do not respond equally to the same stimulus. Capons, however, have the faculty of rapidly regenerating plucked feathers, and in the present series of experiments this fact has been used in order to obtain more uniform results. A dozen or so feathers were removed from an area on the breast, level with the point of the shoulder, care being taken to ensure that the plucked feathers were all solid black and had no irregularities or mismarkings. In about seven days the feathers could be seen pressing the skin outwards but not yet protruding through it, a process which was completed in the next two days. From this stage until about seven days later the feathers were considered suitable for the test.

Technique of Injection of Urine and Examination of Feathers

Ten cc of the prepared urine were injected into the pectoral muscle of the fowl on each of two consecutive days; on the third day feathers (two or three) were removed for examination. With the very young feathers it was found most satisfactory to remove them with a pair of forceps, gripping them transversely and exerting a slow pull in the direction of feather growth, otherwise the feathers were apt to break in the follicle and leave their growing basal ends behind. They were then carefully slit along the side opposite the rachis, the pulp removed and the remaining

feather pressed, inside down, on to a cover slip; as the base is somewhat rounded one or two tiny longitudinal slits are usually made in it in order to make it lie flat. The cover slip is then mounted dry on a slide and the preparation can be examined under the microscope.

Where the concentration of hormone has been sufficient to induce a female reaction in feathers so plucked *the amount of red pigment and its distribution* should be approximately the same in adjacent feathers.

TABLE I

No. of sample	Received	Age of sample days	No. days pregnant	Age years	Mouse test	Capon test	Urine dilution
824	1.8.33	2	148	12	+	++	N
821	30.7.33	3	88	5	+	++	N
996	27.9.33	6	104	10	+	++	N/2
1007	29.9.33	2	101	14	0	0	N/2
991	26.9.33	1	107	12	+	++	N/2
992	26.9.33	1	114	6	+	++	N/2
1006	29.9.33	3	140	3	0	0	N/2
1038	7.10.33	1	166	17	0+	0+	N/2
1074	16.10.33	3	142	11	+	++	N/2
1076	16.10.33	3	172	9	+	++	N/2
1078	17.10.33	3	163	12	+	++	N/2
1079	16.10.33	4	156	22	+	++	N/2
{1084	17.10.33	1	146	2	0+	+	N/2
{1172*	2.11.33	1	162	2	0	0	N/2
{1087	18.10.33	2	133	17	0+	+	N/2
{1165*	2.11.33	2	148	17	0	0	N/2
{1101	20.10.33	3	126	14	0+	+	N/2
{1171*	2.11.33	2	140	14	0	0+	N/2
1170	2.11.33	2	168	13	0+	+	N/2
1162	31.10.33	1	215	8	0	0	N/2
1166	2.11.33	2	255	14	0	0	N/2
1172	2.11.33	1	156	15	0	0	N/2

* Retest.

Permanent preparations of these feathers may be made by drying them, removing the loose scaly parts of the sheath and mounting them in Euparal.

Altogether 22 samples of urine have been analysed and Table I shows the relative data. The close accordance in the results between the capon test and the mouse test indicate that the sensitivity of the former is at least as great as that of the latter. The classification of results under the heading "Mouse Test" in the table refers directly to their use as a

diagnostic property for pregnancy, positive, negative or doubtful. Under the heading " Capon Test " an arbitrary classification of the results has been employed to indicate very roughly not only whether or not œstrin is present in the urine but also the amounts. Since the subject of this paper is a test not for œstrin but for pregnancy in the mare, making use of the fact that large amounts of œstrin are excreted in the urine of pregnancy it will be necessary in a later paragraph, to discuss and define, if possible, the limits of what constitutes a negative and what a positive reaction in respect of pregnancy.

Results Obtained

In good positive reactions such as were obtained in P.F. 1074 and 1076, Plate 10, figs. 5–8, feathers plucked 48 hours after the first injection show an entire absence of black pigmentation along the growing base and up the lateral edges and its replacement by red or yellow pigment. Feathers removed the following day show a widening of the red area, and as a rule some traces of the original black pigment are seen appearing again near the lateral edges. Later pluckings show the melanic pigment increasing in quantity, spreading towards the rachis and so cutting off the red colour in the form of a complete V-shaped band. The characteristic shape of the red bar is determined by the differential rates of growth obtaining in the feather germ; when the feather is fully grown it is no longer V-shaped but flattened out into a more or less straight transverse bar.

The largest bar so obtained resulted from the injection of P.F. 1076 and was about 5 mm wide. Between this type of reaction and none at all, varying grades occurred. Less active samples of urine gave narrower bars, and bars with a break in their continuity between the rachis and the lateral edges, and with a tendency for the greater amount of red pigment to be accumulated round the rachis so that when the pattern could be clearly seen it appeared macroscopically as a red star on the rachis, the quantities of red on the edges being insignificant.

Finally, reactions too weak to be seen macroscopically occurred where only a few red pigment cells appear in the barbs adjacent to the rachis and on the extreme lateral ones. It is only by obtaining uniformity of distribution of the red pigment in different feathers that such slight reactions can be considered positive at all.

In Table I the results have been roughly graded. Those showing no reaction are indicated by (0), and are obviously negative both in relation to the presence of œstrin and to pregnancy. At the other end

of the scale are the reactions which are sufficiently strong to be seen macroscopically; they are designated (++) and from a comparison with the mouse test results it is clear that they indicate the occurrence of pregnancy. Reactions which are not discernible to the naked eye have been divided into two sections—those in which small though regular amounts of red pigment are present (+) and those in which the quantity of red pigment is infinitesimal or is variable in adjacent feathers. The latter can only be regarded as indefinite and are marked (0+). The former clearly denote the presence of œstrin in quantities above that occurring in the normal non-pregnant animals, but not sufficient to allow of positive diagnoses with the mouse test. Samples from four mares yielded this type of result. Regarding one of these (P.F. 1170) no further particulars are available, but new samples of urine were obtained from the other three about a fortnight later. All three retests proved to be negative with the mouse test and two with the capon test, the third producing a doubtful reaction (0+). Of the two which were completely negative one (P.F. 1087 and 1165) was from a very old mare (17 years), and it was subsequently learned that the original sample from the other (P.F. 1084 and 1172) had been taken during an œstrous period. It is known that the excretion of the female hormone in the urine is greater during œstrus and the original positive reaction in this case was therefore to be expected. In the aged mare the reason for the different results from the two samples is not so clear but abnormalities of œstrin excretion with age are known to occur. The only variable in the remaining mare (P.F. 1101 and 1171) which might explain the divergent results was the fact that she was in bad health.

Whatever the explanation of such weak positive reactions it appears clear that they cannot be regarded as an indication of pregnancy when the test is carried out a considerable time after mating. Since the capon tests were undertaken comparatively late in the season cases of early pregnancy were not available for diagnosis, and this aspect of the problem still remains to be examined. Data for the mouse tests suggest that a scale of reactions graded according to the length of gestation would be necessary.

On the Possible Utilization of other Breeds of Fowl for the Detection of Œstrin

All the fowl tests described in the present paper having been carried out on Brown Leghorns it might prove useful to discuss the suitability of other breeds for the purpose. Since the nature of the reaction consists

in the change of the plumage type from male to female, then in all breeds where the two sexes exhibit characteristic differences, it might be expected that the results of injections of œstrin to the castrated male would be similar to those characterizing the Brown Leghorn type of reaction.

So far the only other race of fowls which has been made use of is the Sebright (Freud (1931))—a breed in which there is no sex dimorphism in plumage, the male being hen-feathered. Not only is the structure of the feathers female in form in both sexes but the patterning is also similar; if, however, the Sebright male is castrated the typical fringed feathering of the males of other breeds is developed although except for a decrease of melanin in certain areas of the body the *pattern of the feathers apparently remains substantially as in the uncastrated bird of either sex* (Morgan (1919)).

Freud has found that when such castrated Sebrights were injected with Menformon (œstrin) they produced feathers of the female type on previously denuded areas. According to him this left no doubt that hen-like feathers in birds depend on the female hormone. His further conclusions were stated as follows: "The fact that Menformon treatment replaces the testicles of these animals so far as its influence upon plumage is concerned tends to prove either that the normal Menformon production of their testicles is large or that the susceptibility of their feathers towards Menformon is particularly great."

While there can be no doubt that a female effect can be produced on the plumage of the castrated Sebright male and that such a breed could be used for the detection of the presence of œstrin, a brief review of the facts concerning plumage characterization will reveal the necessity of adopting a cautious attitude at the present time towards the use of breeds other than the Brown Leghorn should the method be brought into general use.

The problem of the hen-feathered male is one that has engaged the attention of many scientific workers. Early investigations on the causation of plumage type in the hen-feathered males, based on an examination of the testes of both hen-feathered and cock-feathered males (Boring and Morgan (1918)) led to the view that the testes of the former possessed definite histological entities (luteal cells) whose function was the same as that of similar cells in the female reproductive gland, namely, the suppression in both of cock-feathering. However, Pease (1921), from an examination of a number of birds formed the conclusion that the amount of luteal tissue in the testis depends on the stage of spermatogenesis reached by the gland and is not associated with the type of the plumage of the bird. That the presence of abundant luteal

tissue does not necessarily give rise to the female plumage was also shown in a castrated male with subcutaneous testis grafts containing much luteal tissue (Greenwood, 1925).

That there is no fundamental difference between the activity of the testes of hen-feathered and cock-feathered males was demonstrated by Roxas (1925) and Greenwood (1928). The experiments consisted in successfully transplanting the testes of a cock-feathered male into a previously castrated hen-feathered male and *vice versa*. Both sets of experiments showed clearly that the endocrine activity of the two-testis types were the same, for if the testes from a cock-feathered male were successfully implanted into a castrated hen-feathered bird the plumage developed was female. The reciprocal experiment led to the same conclusion.

That the differential factor causing the two types of plumage in the male is inherent in the skin and presumably in the feather follicles themselves has been shown by Danforth (1928). He transferred pieces of skin of the saddle region (where sex dimorphism is most marked when present) from a newly hatched hen-feathered bird on to a White Leghorn cock of a similar age. At maturity it was seen that the grafted skin from the hen-feathered bird produced female feathers in spite of the fact that it had from hatching developed on the body of a cock-feathered male whose circulating fluids were alike distributed to his own and to the grafted skin.

Acceptance of all these facts would show that at least the hypothesis that the testis of the hen-feathered male does not differ endocrinologically from that of the cock-feathered male is well founded.

The point that still remains is whether or not the type of feather produced by the hen-feathered male is dependent on the direct action of a female hormone produced by the testis. In this connection it will be necessary to discuss first the action of the secretion of another endocrine gland which is known to produce a modification of the plumage in birds and to determine whether such modifications are of the nature of an apparent change in the sexual type or not. Striking effects in this direction have been induced in experiments in which the thyroid gland has been used. In 1922 Torrey and Horning found that feeding desiccated gland to Rhode Island Red males led to the assumption by these birds of female type of feathering. Similar experiments with other breeds of fowls have confirmed their results. Cole and Hutt (1928), experimenting with birds representing nine different breeds conclude that female feathers could be induced in the males of all these breeds by feeding either raw or desiccated thyroid. One of the breeds—the Silver Wyandotte—was of

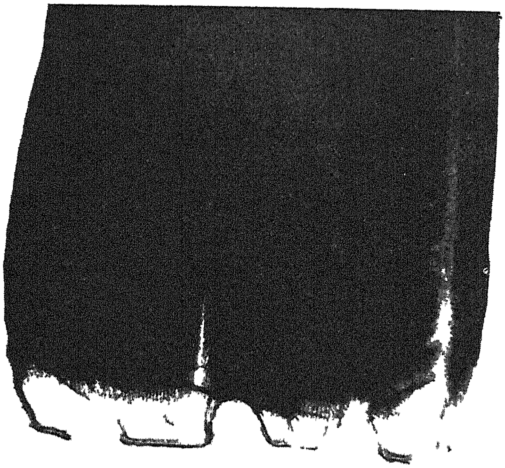

FIG. 1

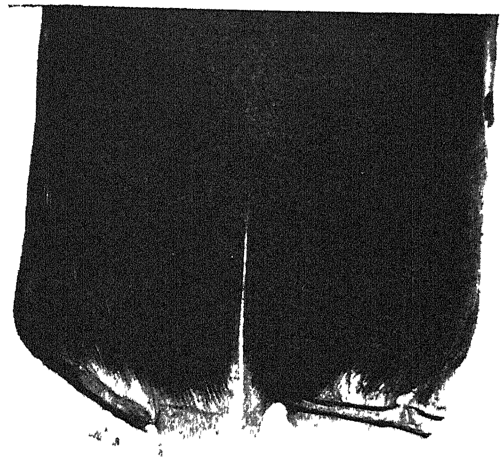

FIG. 2

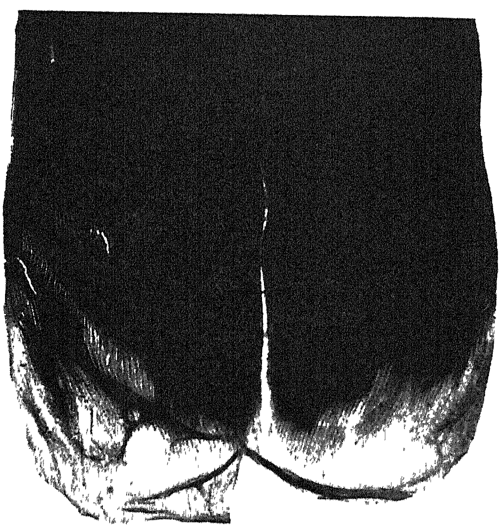

FIG. 3

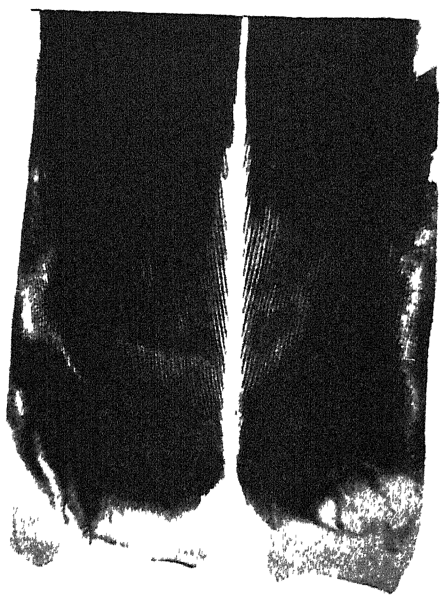

FIG. 4

(*Facing p.* 254)

FIG 5

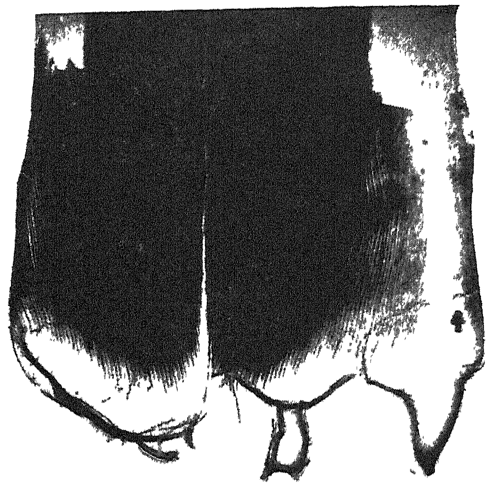

FIG 6

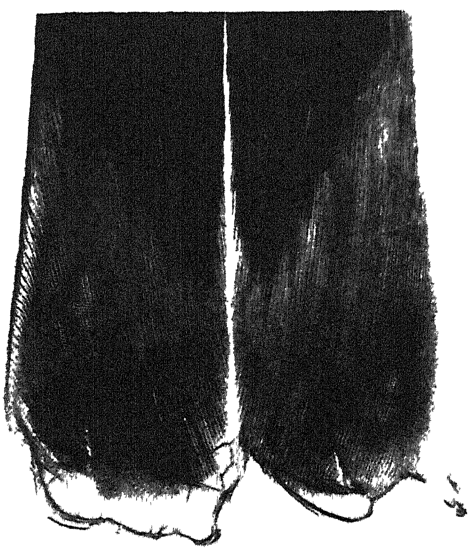

FIG 7

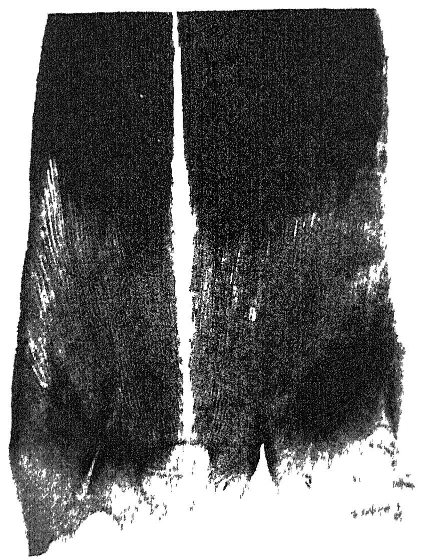

FIG 8

particular interest in that the cocks and hens of this race, like the Brown Leghorn, have feathers different not only in structure but in colour pattern as well ; it was found that the males of this strain responded to the administration of thyroid by producing feathers which were similar to those of the female in colour, pattern and structure. If it could be shown that the Brown Leghorn reacted in a similar way to thyroid medication then it would seem probable that the female type of feathering might not be a direct effect of œstrin on the developing feather follicle influencing or determining its structure and colour pattern, but that this hormone acts indirectly by inducing, by its relations to the thyroid gland, a state of chronic hyperthyroidism.

Cole and Reid (1924) fed four adult male Brown Leghorns with desiccated thyroid gland and found that the new feathers growing in under the influence of this treatment, while not typically female in appearance showed distinct female characteristics. They found that the resemblance to female feathers was much more striking in respect of shape and structure, but as related to colour there was an evident action towards the reduction of red pigment, varying in degree in different birds but tending to be arranged in " stippling " when present. These experiments were repeated on our own stock, but while our results agreed with the former in respect of changes of structure in the feathers from male to female type, it was not possible to confirm the opinion of Cole and Reid that the changes in patterning were suggestive of the pigment distribution found in the normal female (Greenwood and Blyth (1929)). Further, in the breast—a region which the previous authors did not discuss—there were never any indication of a tendency for the black to be replaced by the female colouration (red).

It would seem then that of all the breeds of fowl that have been used for thyroid medication, the only one in which the plumage changes induced are not wholly these which characterize the female is the Brown Leghorn. Experiments in which thyroid gland was removed showed that the operation resulted (1) in a diminution in the amount of melanin and a coincident increase in the amount of red pigment; (2) in an increase in the amount of fringing due to the lack of barbule formation (Greenwood and Blyth (1929)). Hyperthyroidism in the male gives exactly opposite effect. These results, together with what is known from the experiments on gonadectomy led to the formulation of the hypothesis that in the Brown Leghorn the plumage typical of the male is developed independently of the gonad, but that in the development of the female plumage both gonad and thyroid play a part. The former stimulates the latter to a higher level of activity than that present in the male and so indirectly

causes a hyperthyroid effect on the feathers; at the same time it modifies this condition by acting directly on the feathers—restricting the deposition of melanin and so producing the female pattern.

As already mentioned, the typical female type of plumage can be produced by injection of œstrin in suitable amounts. The alteration of shape and structure is by inference a hyperthyroid effect, but it has also been possible to demonstrate that small doses of œstrin simulate in their reaction on the plumage the pigmentary changes induced by a condition of hyperthyroidism (Greenwood and Blyth (1930)). In the Brown Leghorn, then, the two distinct actions of the female sex hormone can be adequately demonstrated. The most convincing proof, however, that œstrin exerts an effect that cannot possibly be obtained by thyroid medication is shown by the reaction of the breast feathers of the capon: they are fully melanic, yet as a result of œstrin injections the development of the melanin can be inhibited and the deposition of red pigment induced.

Having reviewed the evidence that the complete female type of plumage in the Brown Leghorn is induced by œstrin alone and that the female plumage of some other breeds can be reproduced exactly in the male by thyroid treatment, it is now possible to consider the problem of the castrated Sebright as a suitable means of testing the activity of samples of female hormone. It has already been shown that there is no difference in the endocrine activity of the testes from hen-feathered and cock-feathered breeds and the discussion on the effects of thyroid administration leads to the suspicion that possibly in the Sebright one is not dealing with a direct effect of the female hormone on the feather follicles but merely with that phase of the hormone's activity which results in a stimulation of the thyroid gland. If this were true it would not necessarily mean that the testes were capable of secreting female hormone, but merely that the hormone of either sex was capable of causing an effective stimulation of the thyroid gland, sufficient to repress cock-feathers in favour of the development of the female type of plumage. It has been shown clearly that the feathers of the Sebright respond more easily to the stimulus. (There being no evidence relating to the effect of injections of male hormone on the plumage of the castrated Sebright male it is intended to perform some experiments which it is hoped will provide an answer to this question.)

In conclusion then it can be said that of the numerous breeds that have been or might possibly be used for an effective demonstration of the direct action of œstrin on the plumage only the Brown Leghorn fulfils the necessary conditions, since in all other breeds worked with, the development of female plumage in the male may be controlled not

only by the ovarian secretion but also by manipulating the thyroid gland.

Summary

A second method of diagnosing pregnancy in the mare, using the fowl as a test animal, is described. Pigmentary changes in the growing feathers of Brown Leghorn capons ensue as a result of injections of the urine of pregnancy, the reaction being dependent upon the large amounts of œstrin present in the urine at such time.

The test has the advantage of being a rapid one since the diagnosis is available in 48 hours.

The possibility of using other breeds of fowl for this purpose is discussed and the difficulty of distinguishing between the effects of œstrin and of thyroid hormone in many is stressed.

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Explanation of Plates

Plate 9

Fig. 1.—P.F. 1166. Injected November 7 and 8, 1933. Feather plucked 10.11.33. Negative reaction. Melanic granules can be seen below the formed feather barbs.

Figs. 2, 3, 4.—P.F. 992. Injected October 10 and 11, 1933. Feathers plucked on October 12, 13 and 15, respectively.

FIG. 2.—12.10.33. Irregular broken bar of red across base of feather.
FIG. 3.—13.10.33. Complete bar of red across base of feather.
FIG. 4.—15.10.33. Broken bar of red up the feather. Widest in the centre where it is roughly diamond shaped.

PLATE 10

FIGS. 5 and 7.—P.F. 1074. Injected October 22 and 23, 1933. Feathers plucked on October 24 and 26.
FIG. 5.—24.10.33. Marked bar of red across base of feather.
FIG. 7.—26.10.33. V-shaped band of red across feather reaching base centrally and cut off laterally by the return of the black pigment.
FIGS. 6 and 8.—P.F. 1076. Injected October 22 and 23, 1933. Feathers plucked on October 24 and 26.
FIG. 6.—24.10.33. Broad transverse band of red at base of feather and stretching up lateral edges.
FIG. 8.—26.10.33. Very broad red band, black pigment just beginning to appear again at lateral edges of feather.

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The Structure and Origin of Corpora Lutea in some of the Lower Vertebrata

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(*Communicated by W. Bulloch, F.R.S.—Received* May 12, 1934)

[PLATE 11]

1—INTRODUCTION

The bodies known as Corpora Lutea must be considered from several points of view :

(1) their anatomy and histology ;
(2) their development and origin in the individual;
(3) their functions, or influence on other organs, and the effect on them of processes going on in other parts of the body—all of which may be described under the term " physiology " ; and
(4) their etiology, or the causes of their development in the ovary.

Hitherto, and in medical studies still, the subject has been too exclusively considered in relation to the human female, and secondarily in relation to other mammals. Comparatively little attention has been given to other vertebrates. The present paper deals especially with Amphibia and Reptiles.

It is generally admitted that corpora lutea arise from the ruptured follicles of the ovary from which mature ova have been discharged, somewhat similar structures being developed from unruptured (atretic) follicles in which ova have died and are undergoing absorption. Before the appearance of the work of Sobotta on the mouse (1895) there had been considerable controversy concerning the origin of the essential large cells of the corpus luteum in the mammal. Some maintained that they were the enlarged cells of the so-called granulosa or follicular epithelium, others that the latter were discharged with the ovum or absorbed, while the special luteal cells arose from the cells of the theca interna.

Giacomini (1896) stated that the researches of Mingazzini on Reptiles and his own on Elasmobranchs, Amphibia and birds proved that in these vertebrates the follicular cells were not expelled on the rupture of the ovarian follicle, but remained within the follicle as in the mammal according to Sobotta, and took part in the formation of corpora lutea vera even when, as in Amphibia, the ruptured follicle forms no complicated structure, but immediately atrophies and disappears. According to this conception the corpus luteum is merely the ruptured follicle containing the cells of the follicular epithelium, without regard to the changes which occur in the follicle after the escape of the ovum. On this view all ruptured follicles are corpora lutea, and the only corpora lutea falsa to be distinguished would be atretic follicles.

The history of the ruptured follicle was described by Lucien (1903) and Mingazzini in Seps and Anguis which are viviparous lizards, and shown to have a development similar to that found in mammals. A distinct hypertrophy of the follicular cells was described in viviparous Elasmobranchs in *Myliobatis* by Giacomini and in *Spinax niger* by Wallace, while it was found by Bühler (1902) and Cunningham (1897) that the ruptured follicles in oviparous Teleosteans and Cyclostomes began to degenerate immediately after ovulation.

Cunningham (1921) maintained that the essential character of the corpus luteum was the persistence and hypertrophy of the ruptured follicle and especially of the follicular cells which it contains, and that in this sense true corpora lutea according to the published evidence occurred only in viviparous forms among the lower vertebrates, whereas in the

oviparous forms the ruptured follicle showed no persistence or hypertrophy, but immediately began to degenerate and undergo absorption.

Giacomini (1896) published observations on what he called the *corpi lutei veri degli uccelli*, as seen in the domestic hen, showing, as mentioned above, that the follicular cells were not expelled at the rupture of the follicle, and therefore according to his view gave rise to a corpus luteum.

Recently Hett (1922 and 1923) has published detailed and well-illustrated descriptions of the history of the ruptured follicle in the domestic hen and jackdaw. Like Giacomini he applies the term corpus luteum to the follicle in the bird, which, if correct, would be inconsistent with Cunningham's view of the correlation between corpora lutea and gestation or internal development. But our observations on the ovary of the hen show in our opinion that the structure of the follicle of the bird after rupture has not at any stage the essential features of the mammalian corpus luteum to which the name was originally applied. Our general conclusion is that the true corpus luteum, *i.e.*, the persistence and development of the follicular cells within the ruptured follicle, with associated changes in the cells of the internal theca, is the consequence and result of the internal development of the fertilized ovum in oviduct or uterus, or in the cavity of the ovary, and that in oviparous forms the ruptured follicle at once begins to undergo reduction and absorption.

2—Ovulation in Amphibia

Our researches have included experiments on the effect of injections of anterior pituitary extracts on ovulation, and the subsequent changes in the ruptured ovarian follicles. It has been shown by the experiments of numerous investigators on mammals (of which a general account is given by Parkes (1929), chap. IX, that injections of extracts of anterior lobe of ox pituitary have a marked effect in producing corpora lutea. In some experiments when large amounts of extracts were injected, whether simple extract in saline or in N/10 NaOH, all the larger follicles were converted into atretic corpora lutea without ovulation. In others ovulation was produced both in the mature and the immature animal with luteinization of the ruptured follicles.

It was discovered by Hogben and his co-workers (1931) that injection of extract of ox pituitary induced ovulation in the South African clawed toad (*Xenopus lævis*). We have obtained the same results in London, and we have to thank Professor Hogben and Mr. Slome for supplying us with some specimens of *Xenopus* from the laboratory of the London School of Economics.

In *Xenopus*, no signs of luteinization were produced by injection of the extract. This shows that the pituitary hormones have no general property of causing luteinization, but that they merely act as a stimulus to the process of luteinization which is characteristic of the normal mammal without artificial injection. We injected other species of anurous Amphibia without producing ovulation; in fact, in some cases after repeated injection the ovary became reduced and contained smaller ova than in the specimens killed before the injection experiments were begun. The details of these experiments are given below.

(*a*) *Rana temporaria*—Four females, two with much swollen abdomens, ova probably in the oviducts, two smaller and less swollen, ova probably still in the ovaries. The preparation injected was Antuitrin S, supplied by Parke, Davis & Co., extracted from pregnancy urine. In our experiments it was diluted with frog's Ringer solution. Dorsal subcutaneous injections were made into the two smaller frogs on nine successive days.

One of the two discharged its ova on the seventh day.

As the numerous specimens kept without experiment were ovulating naturally, the result of the experiment supplied no distinct evidence of the influence of the injections on ovulation.

In February, 1934, experiments were made of injections of the acetic acid extract of anterior pituitary made by Bellerby's method of the usual strength, one lobe (weight 1·16 gm) to 15 cc of extract—so that 1 cc of the extract corresponds to 77 mgms of gland tissue. A number of small but adult *Rana temporaria* were received on January 31 and the following experiments were made :—

In the first experiment—

January 31—Two females injected with 1 cc of the extract each.

February 1—Both specimens shed their skin in fragments, not entire. One uninjected specimen in the larger aquarium was observed to be doing the same.

February 2—Both frogs injected with 1 cc of the extract a second time.

February 12—One of the two had ovulated.

In the second experiment :—

February 12—Two females were injected as before.

February 14—The same injections were repeated.

February 16—One of the two frogs had ovulated.

In the third experiment :—

February 21—Two females were injected as before.

February 22—One of the two ovulated, and also cast its skin in fragments.

In these experiments the controls had not ovulated.

(*b*) *Rana esculenta*—Four specimens of this species were similarly injected with Antuitrin "S" from March 8 to April 25, but none of them ovulated. After death the ovaries were found to be enlarged, but the ova not mature.

R. esculenta—extract of fresh anterior pituitary of ox. For preparing the extract we used the method of Bellerby (1933), viz. : extraction with 1% acetic acid, and neutralization with 1% NaOH, except that we used brom thymol blue instead of phenol red in the colour test for neutralization. In some experiments we used frog's Ringer solution and in others horse serum for making the extract. The number of specimens injected was seven, the amounts of extracts used from 0·5 cc to 2·0 cc. In some experiments the injections were repeated for 14 or 15 days, once daily. It is unnecessary to give all details since ovulation never occurred. Injection of lamb's thyroid extract in frog's Ringer in addition to pituitary extract was tried, but without result.

(*c*) *Bufo calamita* (natter jack toad)—On May 4, twelve specimens of this species were received from a London dealer. They had the colour characters peculiar to specimens in the south of France, namely, patches of green with red spots and a yellow vertebral stripe on a ground colour of light brown-yellow. One of the females was injected several times with saline extract, and another with acetic acid extract: neither of them ovulated. The first specimen A died on May 22, 11 days after the first injection. The ovaries were very black and of enormous size. It was evident from the distension of the abdomen that none of the specimens had spawned that season. On June 5 all the specimens were found dead, in consequence of the great heat, reaching on the previous day a temperature of 30·5° C.

(*d*) *Xenopus lævis* (the South African clawed toad)—Some female specimens of the above were kindly supplied to us by the staff of Professor Hogben's laboratory at the School of Economics. The following experiments were made :—

Xenopus A

March 28—Injected with 1 cc acetic acid extract of anterior pituitary.

March 29—At 10.30 a.m. was found to have ovulated—128 eggs. This was less than 24 hours after injection. The average diameter of the eggs was about 1·8 mm, of the gelatinous capsule 0·5 mm.

Xenopus A—(continued)

March 30—The ovary preserved in Bouin's mixture for sectioning. The stomach was found distended with eggs which had been swallowed.

Xenopus B

April 10—Was injected with 0·5 cc of the acetic acid extract.

April 11—Had ovulated—1016 eggs counted, besides some adhering to the abdomen of the mother.

April 12—Ovulated more eggs without a fresh injection—200 to 300 by estimate.

April 13—Ovary preserved for sections.

Xenopus C

June 1—Injected with 1 cc of extract of ovary of *R. esculenta* + 1 cc of saline extract of anterior lobe pituitary.

Xenopus D

June 1—Injected with 1 cc of saline pituitary extract only.

June 2—Neither *Xenopus* C nor D had ovulated. Each of them injected again with 1 cc ox pituitary, saline extract.

June 3—*Xenopus* C had ovulated, 359 eggs counted, while D had not.

June 9—*Xenopus* C was killed and the ovaries put into Bouin for sections.

Experiments on the effect of Posterior Lobe.

Xenopus E

June 13—Injected with 1 cc of extract of anterior lobe pituitary in frog's Ringer (15 cc) + 0·5 cc of similar extract of posterior lobe. After about 1½ hours the specimen was covered with thick white mucus, semi-solid in consistency. This effect had been previously observed and recorded by Hogben in 1931.

June 14—Again injected with 1 cc extract of anterior lobe + 0·5 cc of extract of posterior lobe in frog's Ringer.

June 15—No ovulation.

June 16—Ovulation. Apparently extract of posterior lobe delays ovulation.

Xenopus F

June 13—Injected with 1 cc saline extract of ovary of *R. esculenta*.

June 14—Injected with 1 cc saline extract anterior pituitary.

June 15—No ovulation. Injected again with 1 cc anterior lobe.

Xenopus F—(continued)

June 16—Died. Ovaries found to be much reduced with no well-developed eggs. As this was probably the condition at the time of the first injection, ovulation was not possible, and the experiment supplied no evidence that ovulation was inhibited by the ovarian extract.

Xenopus G

June 15—Injected with 0·5 cc saline extract of posterior lobe. White mucus was secreted within half an hour of the injection, but not so freely as with *Xenopus* E.

Xenopus H

June 21—Injected with 1 cc acetic acid extract anterior lobe + 1 cc of Ringer extract of posterior lobe. In a short time it showed a thick white covering of stiff mucus as seen before in *Xenopus* E.
June 23—Injected with 1 cc acetic acid extract of anterior lobe.
June 24—Ovulation—about 10 eggs.

Xenopus I

June 21—Injected with 1 cc acetic acid extract anterior lobe + 1 cc frog's Ringer only. It showed no visible white mucus, evidence that the secretion of mucus is due to extract of posterior lobe.
June 24—Ovulation—about 20 eggs.

Xenopus J

June 23—Injected with 1 cc acetic acid extract anterior lobe only.
June 27—Ovulation—a small number of eggs.

The above experiment (*Xenopus* F) of injecting saline extract of ovary of *Rana esculenta* was made to test whether the absence of response to pituitary hormone in that species was due to a special substance contained in its ovary which would, when injected into *Xenopus*, inhibit the response in the latter species. The converse experiment was also made of injecting saline extract of the ovary of *Xenopus* into *R. esculenta* to see whether this extract would cause the ovary of the latter species to respond to the anterior pituitary hormone.

R. esculenta O (previously injected on May 19 and 22 with acetic acid extract of ox pituitary anterior lobe).

May 26—Injected with 0·5 cc anterior lobe in horse serum + 0·5 cc saline extract from both ovaries of a specimen of *Xenopus*.

R. esculenta O—(continued)

May 29—Same injections repeated.

May 30—Specimen was œdematous, and its skin was perforated with an injection needle—gas escaped. 1 cc of anterior pituitary extract in horse serum was injected.

June 1—Injected with 0·5 cc pituitary extract in frog's Ringer.

June 2—Died. The ovaries were much enlarged, and the eggs well developed.

There was therefore no evidence that the presence or the absence of response to pituitary depended on a separable and transferable substance in the ovary of either *Rana* or *Xenopus*.

MICROSCOPIC STRUCTURE OF RUPTURED FOLLICLES IN *Xenopus*

Three ovaries from which ova had been discharged were fixed in Bouin, and sections prepared from them (1) March 30 about 32 hours, (2) April 13 about 54 hours, and (3) June 9 about 6½ days after ovulation. In (1) the ruptured follicles were still open to the exterior. They were cylindrical in shape with the axis perpendicular to the surface of the ovary, and the base flat, at right angles to the axis. The follicular cells formed an axial column with a space between them and the theca. The follicle was 0·3 mm in length and 0·34 mm in diameter at the base.

The follicular cells had an irregular and abnormal appearance. The cytoplasm was vacuolated, outlines indistinct; nuclei, some vacuolated, others broken up into small particles. The general appearance suggested deformation and absorption.

The thecal walls consisted of connective tissue containing scattered nuclei of normal appearance. The wall contained capillaries with blood, the tissue was vacuolated, and the inner layer formed a dense continuous membrane. The theca containing a large egg approaching maturity was extraordinarily extended and thin. It was better preserved in the sections of the next specimen.

(2) Average length of follicle 0·3 mm, but very much contracted and folded. The average breadth is 0·23 mm. The reduced follicular cells almost fill the cavity in consequence of the contraction, but consist of nothing but vacuolated nuclei, and granular remains of cytoplasm. The thecal wall is of fibrous tissue and capillaries, and its inner layer forming a distinct rather thick membrane deeply stained, which may consist of elastic tissue, fig. 1. In these sections in the large maturing eggs the follicle consists of a single layer of flat follicular cells, and outside

this a very thin layer of connective tissue which contains blood vessels projecting into the surrounding ovarian cavity. All thecæ both before and after rupture are continuous with the external ovarian wall, though they may appear to be free and detached as a result of the section not passing through the region of connection.

(3) Maximum length of discharged follicle 0·2 mm, maximum breadth 0·13 mm, fig. 2. The internal cavity is now quite empty, the follicular

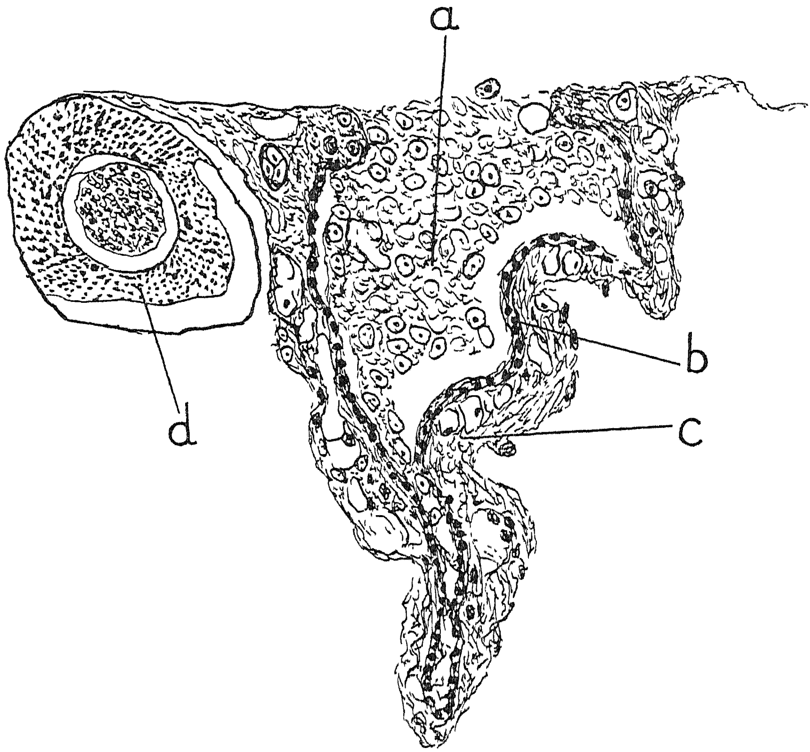

Fig. 1—Ruptured follicle of *Xenopus lævis* about 54 hours after ovulation. *a*, follicular cells ; *b*, theca interna ; *c*, theca externa ; *d*, young ovum. High power

cells having been absorbed with the exception of very few granules. The thecal wall is like a double membrane with a narrow space between, the inner wall being the theca interna mentioned in the previous stages. This wall is continued into a thicker mass of nucleated connective tissue forming part of the ovarian wall where the aperture of rupture, now closed, was situated.

It is evident therefore that reduction and absorption of the ruptured follicle begins from the moment of rupture and proceeds very rapidly.

There is no suggestion of the formation of a corpus luteum, as the term is applied in mammals.

Evidently therefore in anurous Amphibia it cannot be said that the hormone of the anterior pituitary produces corpora lutea, and causes luteinization. It has been shown to induce ovulation in *Rana temporana* and in *Xenopus*, and in the latter case reduction and absorption of the ruptured follicles proceeds directly and in the same manner as in *Rana* after spontaneous normal ovulation.

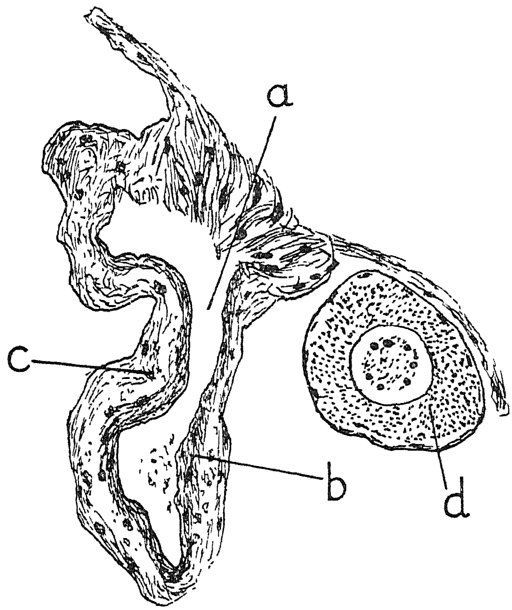

FIG. 2—Ruptured follicle of *Xenopus lævis*, 6½ days after ovulation. Lettering as in fig. 1, except that *a* is the empty follicular cavity. High power

3—Oviparous and Viviparous Reptiles

We have compared the ovaries and ovulation in viviparous and oviparous reptiles, with and without pituitary injections, working chiefly with *Lacerta viridis*, which is oviparous, the Slow-worm, *Anguis fragilis*, and the lizard *Zootoca vivipara*, both of which are viviparous.

(*a*) *Lacerta viridis* (Green lizard)—July 11—Of six *Lacerta viridis* received, body 10 to 12 cm long, tail 20 to 22 cm, two were diagnosed as females from their rather swollen abdomens. One of them, A, was injected with 1 cc of acetic acid extract of anterior pituitary. The next day it was found dead. On examination it had seven large eggs in the right oviduct and six in the left. The eggs were 12·5 to 14 mm long and 8 mm

broad. Prepared and stained specimens of the ovary showed the large ruptured follicles, fig. 6, Plate 11, from which the eggs in the oviduct had escaped. How long the eggs remain in the oviduct after ovulation and before extrusion we do not know, but the ruptured follicle had evidently undergone little change. The aperture was large and the opposite wall of the follicle was invaginated, so that it reached the aperture without protruding from it. On either side of this invaginated wall were two remnants of the follicular cavity full of cells of the follicular epithelium.

It is possible that the injections of pituitary determined the ovulation of the mature eggs, and that it was also the cause of death. The changes in the condition of the follicular epithelium in different stages of the development of the ovum are remarkable. In the earlier stages before the ovum has begun to elongate, the epithelium is remarkably thick. There is a single layer of small cells next to the inner surface of the theca, and internal to this layer is a zone two or three cells thick, composed of large cells with large nuclei, varying in size, not stratified in distinct layers one over the other, but forming one compound layer. As the ovum gets larger, by the accumulation of yolk, the growth of the epithelium does not keep pace with that of the ovum, but becomes more and more stretched until there is finally a single layer of uniform flat cells.

The second specimen (*Lacerta* B) was killed and the ovary showed an advanced stage in the reduction of the ruptured follicles of the last ovulation. The largest eggs present were 2 mm in diameter and still spherical.

July 12—0·5 cc acetic acid extract was injected into two of the surviving specimens, *Lacerta* C and D. On the next day one of them had shed three large and perfect eggs. Both C and D were again injected with 0·5 cc of acetic acid extract anterior pituitary.

July 18—*Lacerta* C extruded two more eggs, and on the 19th was found dead. No eggs were present in the oviduct, two discharged follicles were recognized on one side, and three on the other. In this case also the pituitary seems to have caused the ovulation and probably the subsequent death. *Lacerta* D was then killed and dissected. There were no nearly mature eggs in the ovary, the largest being 4 mm in diameter, and beginning to become oval. The ovary was vascular and congested. In this case ovulation was not to be expected, the most advanced eggs being too immature. Two sets of maturing eggs were recognized, and two sets of follicles from the two last ovulations, six showing apertures or depressions where the rupture had closed, six appearing as minute yellow spots. Thus four successive ovulations were indicated, two in the future and two in the past.

Sections of this ovary showed the structure of the two stages of the degenerating follicles. In the more recent, which may be indicated as Stage 2, fig. 7, Plate 11, the follicle contains a large central cavity which is bounded by a single layer of giant cells, somewhat resembling a columnar epithelium. Within the cavity there are a few small cells, irregularly scattered, embedded in granular coagulum, and in places, chiefly at the narrow end of the section, there are curious convoluted fibres which have a homogeneous hyaline appearance, like threads of gelatine. These take up eosin dye and do not seem to be part of the organic structure. By careful examination it was found that this colloidal substance lay between the outer ends of the giant cells, and the layer of small cells external

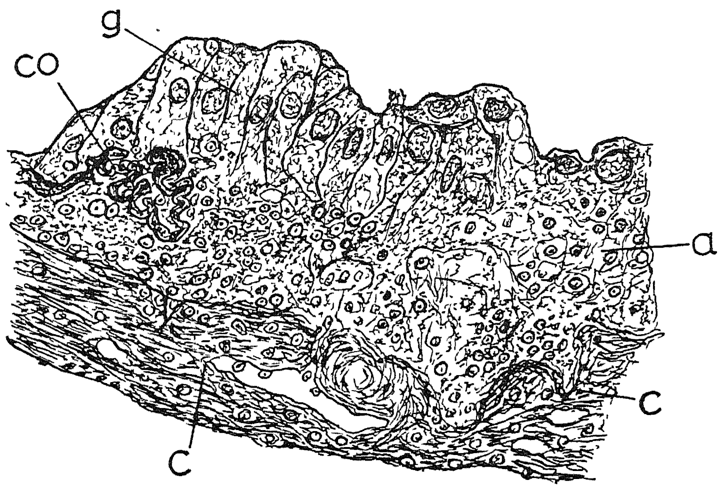

FIG. 3—Portion of section from same series as fig. 7, Plate 11, under high power. *a*, follicular cells ; *g*, giant cells ; *co*, colloid substance ; *c*, theca externa

to them. In one or two places it was traced radially between the giant cells into communication with the central cavity, but it never retained its coagulated appearance within that cavity. It seems evident that it is a secretion given out by the giant cells, which is coagulated by the fixing reagents. This secretion is apparently dissolving the cytoplasm of the smaller cells, the nuclei of which are seen, not yet dissolved, between the twisted cords of the colloid. There can be little doubt that the giant cells have the function of digesting and destroying the original follicular cells. They originate in the centre of the follicle, perhaps by the enlargement of the follicular cells. These giant cells have large granular nuceli, with usually one or two distinct nucleoli, fig. 7, Plate 11.

Outside the layer of giant cells there is a narrow zone of small cells, with small nuclei, which are obviously the remainder of the original

follicular cells. Some of them are distinct and separated, but here and there are rounded granular masses containing numerous small nuclei, 10 to 15 in number. They resemble those of the small cells of the outer layer, and the masses may have been formed by the fusion of the cytoplasm of the small cells, or by the envelopment of small cells by the cytoplasm of the giant cells. The latter supposition is, however, improbable, because there is no large nucleus present in the mass. A third possibility is that they are giant cells in which the single large nucleus has divided up into a number of small ones, but the circular outline of the masses is against this supposition. It is noticeable that the small nuclei have a circular arrangement near the periphery of the cytoplasmic mass.

The theca interna is not distinct from the peripheral layer of small cells, but in places it is seen to be encroaching upon the theca externa, especially on the outer side where the latter is continuous with the wall of the ovary. Otherwise the theca externa is still a rather thick layer of fibrous tissue with blood vessels. Over the outer surface of the theca the ovarian or germinal epithelium is distinctly thickened, and stains intensely. In one series it was impossible to measure exactly the area of sections through the middle of these degenerating follicles on account of their crescentic shape. The maximum length along the chord of the arc of the curve was 1·46 mm and the maximum breadth of the structure was 0·74 mm giving an area of 1·08 sq mm. The actual area would be somewhat greater. The largest section in the series from which fig. 7, Plate 11, was taken measured 1·57 mm in maximum length and 0·75 mm in maximum breadth, and as the section was not curved it would be within a rectangle of these dimensions having an area of 1·17 sq mm.

The third stage of degeneration in our series appears to be that of a female found dead, but quite fresh, on July 24. The ovaries were large and contained a number of maturing eggs, and several distinctly orange-coloured follicular bodies. The largest of the latter in sections were 1·02 mm maximum length, 0·35 mm maximum breadth, or 0·35 sq mm in area. This is a great reduction in size in comparison with the preceding stage, and as the maturing ova were rather smaller than those of the second stage instead of larger it may be concluded that this specimen was not preparing to ovulate again, but was probably passing into the inactive condition with respect to breeding.

A stage almost exactly similar occurs in the same ovary which contained Stage 2 of the degenerating follicle above described. In section, these examples of Stage 3 measured 1·03 mm in length and 0·38 mm in maximum breadth, so that there was no significant difference in size

from those described in the preceding paragraph. As the ovary in which they occurred was preserved immediately after the animal had been killed, the histological structure is more perfectly preserved. The interior consists of a compact mass of cells transversed here and there by capillaries, with no central cavity. The cells are of two kinds, both with indistinct outlines. The majority have small round nuclei with one central nucleolus. Here and there are larger nuclei (more than twice the size), also with central nucleoli, more faintly stained than the others. These cells with large nuclei are evidently the giant nuclei of the earlier stage, now much reduced in size. The fibrous tissue of the theca has almost disappeared, and is replaced by cells, except on the outer wall connected with the surface of the ovary, where some fibres can still be seen. The middle of this wall, in the positions where the rupture occurred, originally projects beyond the general surface of the ovary, and as usual the germinal epithelium over this region is thickened.

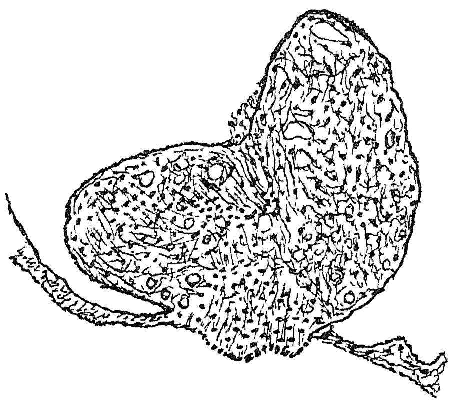

FIG. 4—*Lacerta viridis*, late stage of degenerating follicle. Low power.

The last stage in our series, Stage 4, occurred in a specimen killed on July 7, which had never been injected, fig. 4. The largest developing ova were about 2 mm in diameter and spherical. The reduced follicles in sections were 0·51 mm by 0·32 mm, but this may be only the transverse section, and the longitudinal diameter may have been greater. In this stage the large nuclei have entirely disappeared, the fibrous theca is scarcely to be distinguished, and the whole structure is a nodule of cellular tissue with small nuclei and numerous vacuoles, while blood vessels are small and scarce.

(*b*) *Anguis fragilis* (Slow-worm)—We obtained a number of living slow-worms from the beginning of May to the end of August. They were collected in a locality to the north of London, by one of the keepers

in the Zoological Society's Gardens, Regent's Park. We found it very difficult to distinguish the sexes from one another. It was noticed that many of the larger specimens had short blunt tails, and others long tapering tails. We thought the former were males and the latter females. Mr. Parker, of the Natural History Museum, showed us that the short blunt tails were merely those which had been broken off, and begun to regenerate at the tip. Afterwards we concluded that the snouts of the males were broader and blunter than those of the females, but the difference was so slight that it was not always possible to be sure of distinguishing the sex of an individual. The extrusion of the copulating organs of the male required too much force to be applied to specimens which it was desired to keep alive and uninjured.

Injection of pituitary extracts was carried out to find whether it was possible to induce ovulation in unfertilized individuals, but as the ova are not normally extruded in this species from the oviducts it was usually impossible to know when ovulation took place without killing the specimen.

The earliest stage of the ruptured follicles after ovulation was found in a female killed on June 9. There were four ova in one oviduct and three in the other, the eggs being 9 mm in length and 7 mm in breadth. It was not possible to distinguish embryo or blastoderm in the eggs with the naked eye, but microscopic preparations showed that there was a very early blastoderm. The ruptured follicles were visible in the ovaries, and in prepared sections showed a condition very similar to that described above in the earliest stage of the ruptured follicle in *Lacerta*, fig. 6, Plate 11, the chief difference being that the invagination of the base of the follicle does not extend so far towards the aperture of rupture. The sections are transverse to the long axis of the follicle and to the corresponding axis of the aperture of rupture. The latter is widely patent, the follicular cells form a compact mass which is exposed at the aperture with no signs of separation or loss of any of the cells. The maximum transverse diameter of the follicle is 1·9 mm, *i.e.*, about 5/18 of the diameter of the follicle containing the mature ovum. The thickness of the follicle was about 1 mm and that of the theca 0·25 mm.

The follicular cells have oval nuclei usually with one, or rarely two, distinct nucleoli, and the boundaries of the cells are indistinguishable. The mass is limited externally by a basement membrane which adheres to the cell-mass, but is separated by contraction from the theca interna leaving a cavity which contains here and there a few detached cells. This cavity is enlarged opposite the aperture where it contains granular serum and a clump of red corpuscles. A similar but larger space occurs

on the left side of the section, having similar contents. The theca interna consists of numerous cells and a small amount of fibrous matrix, and contains a zone of small blood vessels near the inner border. The blood in the cavities between theca and follicular cells is derived from the rupture of these small vessels. The theca interna is about 1/3 of the thickness of the whole theca. The external theca shows dense fibrous tissue with scattered nuclei, and contains a zone of very large blood vessels. The external epithelium of the ovary is considerably thickened over the theca near the edges of the aperture.

The second stage shows the corpus luteum fully developed, and contrasting strongly with the second stage of the follicle in *Lacerta.* This occurs in a specimen killed on June 14, which was 34 cm long, the tail being 18·5 cm. It was injected on June 7 with 0·5 cc extract of anterior pituitary in frog's Ringer (one anterior lobe to 15 cc of extract). On June 12 it discharged four large ova. They were far too dry when found to make out whether they contained a blastoderm or not. When killed five more ova were found, three in the left oviduct and two in the right. Each had a small white disc, which seemed like an unfertilized blastoderm. On subsequent examination by microscopic sections it was found to contain an early embryo showing the beginning of the formation of the spinal cord. This was the only case in which discharge of ova from the oviducts was observed in *Anguis.* It may have been the effect of injection of pituitary, but several others were injected without producing this result, so that not much importance can be attached to it.

The corpus luteum in this stage shows the closing of the aperture by the meeting of the inturned edges. The maximum diameter of the sections is 1·48 mm and the transverse diameter 0·42 mm. It is evident therefore that there has been considerable contraction, but this is to a great extent due to the obliteration of the cavities and diminution of the large blood vessels seen in the preceding stage. There is little change in the internal mass of follicular cells; the basement membrane is less distinct and there is no space between the boundary of the follicular cells and the theca interna; and the whole structure is less vascular.

The third stage was seen in a specimen which died on July 12. The blastoderm covered half the yolk longitudinally and the embryo was well defined but slender, and the tail just beginning to coil up. There were three eggs in each oviduct.

The corpora lutea were rather larger than in the previous stage. Of two measured in one section, the larger was 1·5 mm in maximum length and 1·08 mm maximum breadth, and the other was 1·4 mm maximum

length and 0·7 mm maximum breadth. It is possible that some of the eggs had been expelled from the oviduct, as four corpora lutea were seen in sections of one ovary. The structure was similar to that of the previous stage, but the following points were noticed: The basement membrane enclosing the mass of follicular cells was in places more distinct, scattered erythrocytes were seen in the outer zone of the follicular mass, and also in the theca interna. The theca externa in the part connected with the external wall of the ovary was loose and vacuolated, not compact as in the internal part of the theca. There were several small immature ova in the ovary, five being counted in one section.

Later Stages—The stages in *Anguis* are distinguished as those of the development of the embryo, since the date of fertilization is unknown. On June 29 a large specimen was killed which had never been injected and had a truncated tail with a regenerated point. It has seven eggs in the right oviduct and six in the left, each with a well-developed embryo, with heart beating, tail long and curled up, and the area vasculosa covering half the ovum. The ovaries had numerous small eggs and much black pigment. The maximum length of the corpus luteum was 1·3 mm maximum breadth 0·6 mm.

In another female found dead on July 24 the embryo was a little more advanced, the body 1·4 cm long, the tail curled but too rigid to be measured, and the blastoderm completely surrounding the yolk. The ovaries were much decreased in thickness and contained only small immature eggs in addition to corpora lutea. There were 11 embryos, 6 in the right oviduct and 5 in the left. One corpus luteum measured by the stage micrometer was 1·23 mm in maximum length, 1·0 mm in maximum breadth. In structure there was little change. The line of demarcation between follicular cells and theca interna was in most parts indistinct, but in two places invasion of internal theca cells with scattered erythrocytes could be traced into the interior of the mass of follicular cells.

The most advanced embryos were found in a specimen killed on July 20. Like that of June 29 it had a truncated tail. The body of the embryo was 1·5 cm, the tail 1·4 cm, total length 2·9 cm. The maximum length of the corpus luteum, fig. 5 and fig. 8, Plate 11, was 1·05 mm and 0·71 mm in breadth. In both these last stages, especially the last, the thickening of the germinal epithelium on the external surface of the ovary over the outer surface of the corpus luteum was very conspicuous; it formed a regular columnar epithelium.

In all these three specimens showing the oldest corpora lutea, the age being estimated by the stage of development of the embryos in the oviducts,

the reduction in the total volume of the ovary was remarkable. The organ was scarcely more than 3 mm in thickness, there were few ova, and these only in an early stage of development with thick follicular epithelium, with a small number of primitive minute ova near the ovarian surface. In many sections of the most advanced stage there are no ova except the minute primitive stages, but only two large corpora lutea. Sections of an atretic follicle in process of degeneration also occur. These features indicate the complete inhibition of ovular development during the development of the embryos in the oviduct, associated with

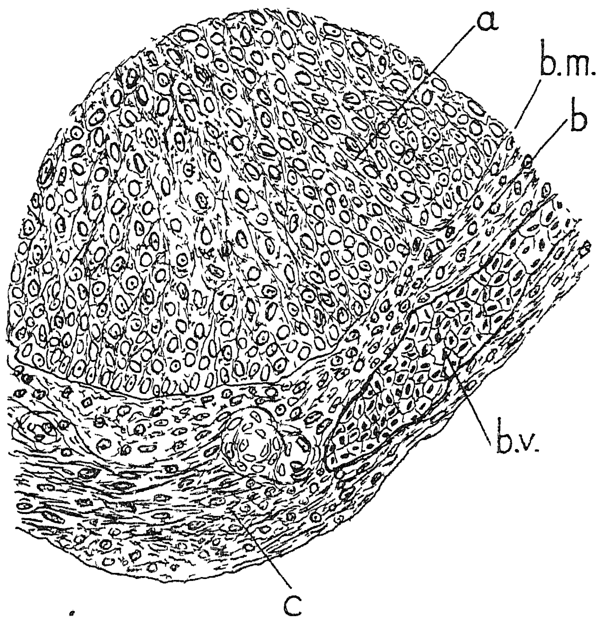

FIG. 5—Lower right-hand portion of section shown in fig. 6, under high power. *a*, follicular cells , *b*, theca interna ; *c*, theca externa ; *b.v.*, blood vessel ; *b.m.*, basement membrane

the persistence of the corpora lutea. The question arises whether the persistence of the follicular bodies, which is commonly described as the formation of corpora lutea, and the associated inhibition of egg maturation are directly due to substances absorbed from the pregnant oviduct, or to such absorption acting primarily on the pituitary and only indirectly on the ovary. There is not sufficient evidence at present to decide this question, but it seems to us that the direct action is more probable.

(*c*) *Zootoca vivipara*—(the English viviparous lizard)—Twelve specimens of this species were received on May 26, 1933. In these there was no difficulty in distinguishing the sexes. In the male the abdomen

was of a deep orange-brown colour, while in the female it was only slightly yellowish brown. The snout of the female was slightly more pointed than in the male, as in *Anguis*.

Two females were isolated and injected with anterior pituitary extract in horse serum, 0·25 cc or 0·5 cc into each, on five days between May 26 and June 5. On the last date one of these females was found dead, and the other was killed. The latter had five large eggs in each oviduct, and sections of these showed a very early stage of the embryo which as the females were isolated on May 26 must have been at least 10 days old. These were therefore fertilized before the injections, and the pituitary extract could have had no effect. The sections of the ovary of the specimen killed on June 5 showed the early stage of the follicular body, with the aperture of rupture almost closed. Another specimen died on June 13. It had four large eggs in each ovary, but none in the oviducts. No follicular bodies were visible in the ovaries.

The stage of the follicular body on June 5 is distinctly later than that of *Anguis* of June 9 (*Anguis*, stage 1) and closely agrees with *Anguis*, Stage 2). In both these cases the mass of follicular cells is separated from those of the theca interna by a basement membrane, and is not penetrated freely by vascular tissue as in the mammal, *e.g.*, rat. In the latter the corpus folliculare has no central cavity as in the human species, and is much larger than the nearly mature ovarian follicle with its large cavity from which it arises. This difference in volume is due to the fact that about half of the tissue of the follicular body in the rat consists of vascular tissue containing numerous capillaries, the nuclei of whose walls are numerous and conspicuous from their elongated fusiform shape, and deep staining in hæmatoxylin. In the mammal, too, the theca externa is scarcely distinguished from the general ovarian stroma.

Thus although we had no complete series of stages in *Zootoca* as in *Anguis* we have no doubt that the follicular body in the former persists during gestation, as in the latter.

4—Discussion

Corpora lutea are developed from ruptured ovarian follicles, after the escape of the ova. When the ova are discharged from the body immediately after leaving the ovary, either in the Amphibia in which fertilization is external, or in oviparous reptiles in which the ova are fertilized in the oviduct but do not remain there during development of the embryo, the ruptured follicles at once begin to undergo reduction and absorption. These processes are more complicated in oviparous reptiles where the

ova are much enlarged by the accumulation of yolk, and the follicles are enlarged in proportion. When development of the embryo goes on in the oviduct, *i.e.*, in viviparous reptiles, the ruptured follicles persist and increase in size. This increase is due to the enlargement of the cells of the follicular epithelium, which do not escape with the ovum; not to increase in the number of cells, since mitoses are not observed. The so-called corpus luteum therefore is a ruptured follicle whose reduction and absorption are delayed until the end of gestation, or until an advanced stage of that process.

With regard to the evolution of the corpus luteum, it must be concluded that its development is directly due to the presence of the developing ovum in the oviduct, and that it is therefore merely the consequence of the evolution of gestation. The presence of the embryo also has important effects upon the structure and functions of the wall of the oviduct. These effects reach their maximum in the mammals. In viviparous reptiles where the egg is enclosed by a vitelline membrane and the nutrition of the embryo is derived from the yolk contained in the ovum, the wall of the oviduct supplies little more than the oxygen for the respiration of the embryo.

The present state of knowledge points to the conclusion that the persistence of the follicle, and especially of the follicular cells it contains, is due to hormones derived from the developing ovum. The follicle and its epithelium continue alive and active while the ovum contained within them is alive and developing. When the ovum dies, a frequent occurrence in the normal life of the ovary, the follicular cells absorb the ovum. In mammals where there is no yolk, the follicular cells die soon after the death of the ovum, and an atretic follicle is formed of which the cells of the theca interna form the principal part. When the ovum continues to live and develop in the oviduct the follicular cells form the corpus luteum.

With regard to the maintenance of pregnancy as one of the four functions of the corpora lutea, the experimental evidence is contradictory (see Parkes, 1929, p. 196). If the function exists it can only be reciprocal, for it is certain that the termination of pregnancy in the mammal is followed invariably by the atrophy and absorption of the corpus luteum. It must be concluded therefore that the hormones absorbed from the pregnant oviduct or uterus are the cause of the full development and persistence of the corpus luteum. In the latter half of pregnancy the body begins to diminish and it is probable that this is due to the fact that in the more advanced stages of fœtal development, the substances absorbed from the fœtus are different.

The inhibition of ovulation as a result of gestation and the formation of corpora lutea have already been discussed.

It is evident that the sensitization of the uterus for the implantation of fertilized ova is a function of the corpora lutea which can only exist in mammals, and in other vertebrates which develop some degree of attachment of the embryo to the uterine wall. It cannot occur in viviparous reptiles like *Anguis* where the ovum is separated from the epithelium of the wall of the oviduct by a vitelline membrane. According to the theory that the presence of the ovum, and the embryo developed from it, was the stimulus which caused hypertrophy of the uterine wall (a stimulus much greater in the mammal by reason of the loss of the yolk and its vitelline membrane), and that this hypertrophy became hereditary in the presence of the hormones which were in action during its evolution, the occurrence of the hyperthropy in association with ovulation and œstrus in mammals would be explained. It is to be concluded on this theory that the hypertrophy of the uterine wall has in the course of evolution occurred at an increasingly earlier stage, so that it now anticipates the presence of the stimulus which originated it, a precocity of development which occurs in the mammary glands and many other cases. Thus the uterine cycle is to a certain degree repeated at every œstrus, and every menstruation in the human subject. This incipient hypertrophy in the majority of cases soon breaks down in the absence of fertilization. In the mammal the corpus luteum is influenced by absorption of substances from the hypertrophied uterine wall as well as from the developing embryo, and is thus influenced at the time of œstrus when fertilization does not occur. In pseudo-pregnancy whether natural as in the dog, or experimental, the hypertrophy of the uterus persists for a longer or shorter period, and the corpora lutea in the ovary also persist in correlation. The persistence of corpora lutea in mammals during pseudo-pregnancy in the absence of fertilized ova is therefore not inconsistent with the theory that their development was originally due to the presence of the developing embryos in the oviduct.

The fourth function attributed to corpora lutea is the development of the mammary glands. As there are no such glands in any class of vertebrates except mammals, while corpora lutea occur in viviparous reptiles and fishes, it is obvious that the relation of corpora lutea to mammary glands has arisen in the course of the evolution of these glands in mammals. Here again the facts become intelligible if we suppose that the evolution of the glands was primarily due to an external stimulus, namely, the sucking action of the young in trying to obtain nutriment from the mother's skin, and that the heredity of the effects only manifests

itself in the presence of the hormones which accompanied the hypertrophy due to the external stimulus.

Similar reasoning applies to the action of the anterior lobe of the pituitary. This term though generally used is obviously unsatisfactory. The word pituitary was founded on an idea long since known to be incorrect, and in development and function the so-called anterior lobe is quite independent of the posterior. Since the former develops as an outgrowth of the oral invagination termed the stomodæum, it seems more appropriate to use the name *stomodæal gland* for anterior lobe, as being more precise and definite. It is difficult to understand why this gland should have any primary connection with the periodicity of the ovary, any more than with the function of the testis. It is known that an association between tumours or other abnormalities and sexual precocity is not confined to the pituitary, but also occurs in the case of the adrenal. Experiment shows that the injection of large amounts of extracts of the stomodæal gland produces ovulation in frogs, as recorded in this paper, and in mammals. It is not, however, a primary or general property of the secretion or extract to cause any other changes in the ovary or oviduct. Its influence on the formation of corpora lutea in mammals and probably in other viviparous vertebrates is therefore secondary. The effect is not due to a change in the secretion, but is a new response arising in the course of evolution as a consequence of gestation, and this response can be explained by the theory already mentioned, namely, that the effect of the stimulation due to the eggs or embryos in the uterus was produced in the presence of the special secretion of the gland in question, and, becoming hereditary, is manifested in the presence of an excess of the secretion even in the absence of the original stimulus.

Similar considerations apply to the influence of the secretion of the stomodæal gland on the development and secretory activity of the mammary glands. In some of the tail-less Amphibia the hormone of the stomodæal gland influences ovulation, but corpora lutea and mammary glands are absent. In oviparous reptiles we find corpora lutea developed as the effect of gestation, and in mammals the injection of pituitary extract determines ovulation and the formation of corpora lutea, the discharged follicle in this case being not merely the follicle without the ovum but taking on a more complicated structure resembling that of the corpus luteum of pregnancy. In the mammal mammary glands have also been evolved, and their development and activity are new responses to the hormones of the stomodæal gland which are present in the lower vertebrates, and doubtless also present in the ancestors of the mammals before the mammary glands were evolved. Although it is not possible

at present to explain completely the hormonic control of lactation, the theory that the evolution of the mammary glands was primarily the effect of the external stimulus of sucking by the young, and took place in association with hormones from three principal sources, namely, the uterus and fœtus, the ovary (corpora lutea) and the stomodæal glands, agrees with the chief facts known at present concerning the relations of these organs and the mammary glands.

The expenses of this research have been defrayed partly by a grant from the Royal Society Government Grant (J. T. C.) and partly from the London Hospital Medical College Research Fund (W. A. M. S.).

5—Summary

In *Rana temporaria* injection of a commercial preparation of gonadotropic hormone from pregnancy urine was not found to induce ovulation, but injection of acetic acid extract of stomodæal gland (anterior lobe of pituitary body) of the ox, in February, 1934, was followed by ovulation, when the controls had not ovulated.

In *Rana esculenta* no ovulation occurred either after injection as above, or with acetic acid extract of ox anterior pituitary, or without injection.

In *Bufo calamita* (the natter-jack toad) injections were made without result.

In *Xenopus lævis* positive results of experiments on ovulation were obtained and sundry other experiments recorded.

The structure of the ruptured follicles in *Xenopus* at three successive stages after ovulation is described, and it is shown that reduction and absorption of the empty follicle begins immediately after rupture and proceeds rapidly, no true corpus luteum being found.

The history of the ruptured follicle in the oviparous *Lacerta viridis* is compared with its history in the two viviparous forms, *Anguis fragilis* (slow worm) and *Zootoca vivipara*. The follicle immediately after rupture in the one case is very similar to that in the other, a collapsed sac with wide aperture, containing the follicular cells. In *Lacerta* the aperture closes and the mass of follicular cells begins to undergo absorption and reduction. In *Anguis* and *Zootoca* the closed follicles persist without signs of absorption during the development of the embryos in the oviduct, forming a structure essentially similar to the corpus luteum of the mammal.

It is concluded that the fertilized ova within the oviduct give off substances which are absorbed by the blood and act as hormones which

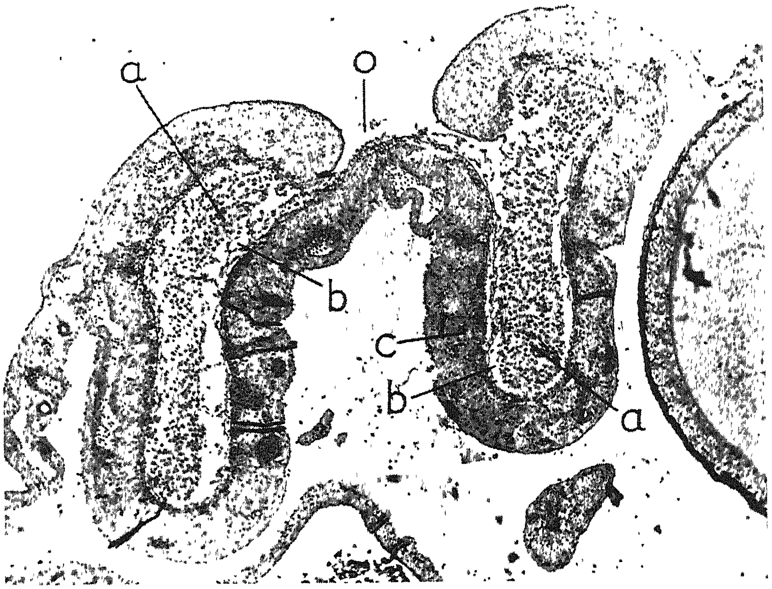

FIG. 6

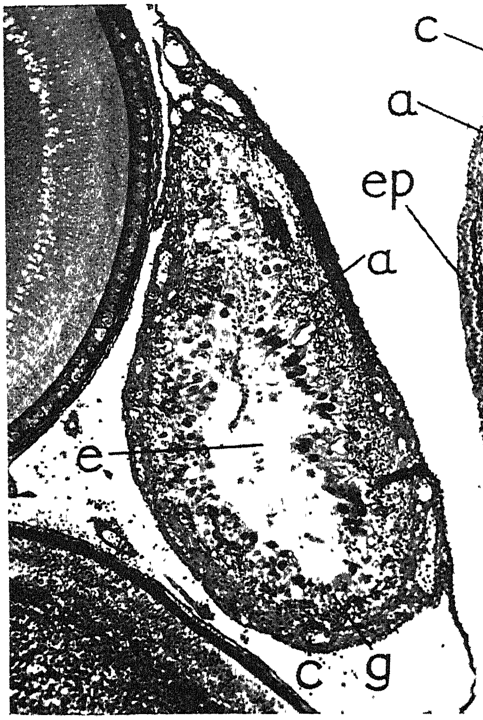

FIG. 7

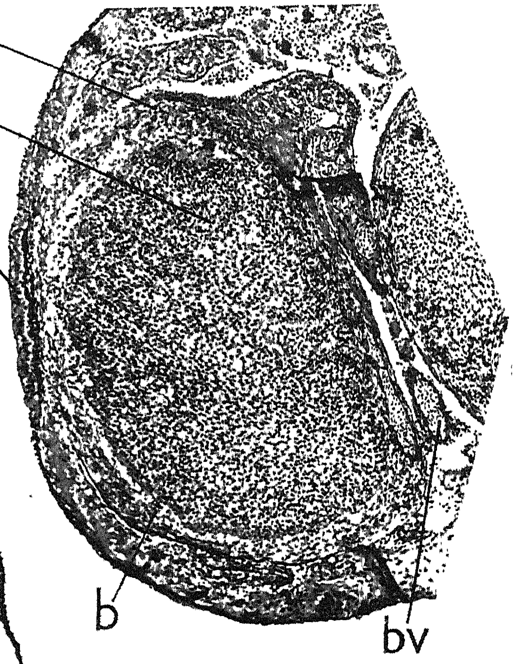

FIG. 8

(*Facing p.* 281.)

cause the persistence of the discharged follicles as corpora lutea, and that these hormones cause either directly, or indirectly through substances given off from the corpora lutea, the inhibition of ovular development and ovulation.

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Description of Plate 11

FIG. 6—*Lacerta viridis*, ruptured follicle (low power). *o*, aperture of rupture ; *a*, follicular cells ; *b*, theca interna ; *c*, theca externa.

FIG. 7—*Lacerta viridis*, degenerating follicle after ovulation, stage 2 (low power). *g*, giant cells ; *e*, central cavity. Other letters as in fig. 6.

FIG. 8—*Anguis fragilis*, corpus luteum in specimen of which the oviducts contained embryos about 2·9 cm in length (low power). *bv*, blood vessel ; *ep*, germinal epithelium. Other letters as in fig. 6.

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The Absorption of Simple Lysins at Cell Interfaces

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(*Communicated by Sir E. Sharpey Schafer, F.R.S.—Received June* 18, 1934)

The existing theory for the action of hæmolysins treats the phenomenon as the result of an irreversible reaction between the lysin and some component of the cell membrane, this leads to a loss of semi-permeability of the cells, which are supposed to have different resistances to the destructive effect of the lysin. Up to now the justification for this theory is that the equations in which it is expressed describe time-dilution curves and percentage hæmolysis curves satisfactorily, but no attempt has been made to follow the changes in lysin concentration which accompany the lytic process. So far as the present theory is concerned, these might be of three kinds. (1) The concentration of free lysin in the system should fall as a result of the lysin's combining with the cell membrane. (2) During the process of lysis, hæmoglobin and other cell contents are liberated, and these may react with the lysin, producing either inhibition or acceleration. (3) On the addition of the lysin to the cells, some of the former may be " absorbed " or " fixed " at the cell surfaces, just as sensitizing agents and complement are " absorbed " (see Ponder, 1932, *a*, 1933). Further, the quantity so absorbed may change as time goes on.

This paper is concerned with some of these questions.

1—" INITIAL " ABSORPTION

Some simple lysins are absorbed in large amounts so quickly that the absorption is virtually instantaneous. Such absorption I call " initial," in order to distinguish it from the slower " delayed " absorption to be described directly. This initial absorption can be demonstrated in the following way.

A time-dilution curve is plotted for 30% lysis for the lysin under consideration, the Stuphenphotometer being used to give the end-point (Ponder (1932, *b*)). The systems are prepared by putting 1·6 cc of the various dilutions of the lysin into a series of tubes, adding 0·8 cc of saline, and then removing 1·6 cc of the mixture into the glass cell of

the photometer. The time for 30% lysis of 0·4 cc of standard cell suspension (rabbit or human red cells) added to the system is measured. The range over which measurements should be made is from 0·1 minute to about 10 minutes. The time-dilution curve can be obtained with extraordinary accuracy in this way.

A second series of tubes is now prepared. Each contains 1·6 cc of the various dilutions of the lysin. The tubes are placed, one at a time, in a container on a specially designed centrifuge revolving at about 10,000 r.p.m. (see Ponder (1932, *a*)) ; 0·8 cc of standard suspension is added, and the centrifuge is started immediately. After 10 seconds it is stopped, and in this time all the cells should have been thrown down. As quickly as possible 1·6 cc of the supernatant fluid is removed and placed in the glass cell of the Stupho, and, when the fluid has reached the temperature at which the experiments are carried out (usually 25° C), 0·4 cc of the standard suspension is added. The time required for 30% lysis is determined as before. In this way we obtain a second time-dilution curve, and, from its relation to the first, the quantity of lysin absorbed from each dilution of the lysin can easily be found.

It is very important that the experiment be done in this way, and in such a manner that the standard system and the system subjected to centrifuging are treated identically as regards being placed in tubes, transferred by pipettes, etc. The reason for this is that many lysins are "adsorbed" on glass, and if a system is pipetted from vessel to vessel oftener than is the standard with which it is compared, as much as 10% of the lysin may disappear by reason of its being taken up on the walls of the vessel or pipette.

The range over which the measurements of absorption can be made is limited by two factors. If the concentration is too great lysis may occur during the 10 seconds spinning. Tubes which show any hæmoglobin in the supernatant fluid must be discarded. If the dilution of lysin is too great, 30% lysis may not be complete within 10 minutes or so. It is inadvisable to extend observations to longer times because of the "spontaneous changes in light transmission" remarked upon by Kesten and Zucker (1928). It is true that such changes are small at 30% lysis (Ponder, 1932, *b*)), for which reason 30% lysis, rather than a lesser amount, is chosen, but it is better to run no risks.

By its very nature, this method measures only initial absorption, complete within 10 seconds. The results can be stated briefly, for they are so definite that there is no point in giving them *in extenso*.

1. When initial absorption occurs, the quantity of lysin absorbed is always a constant fraction of the quantity initially present, at least over

the greatest range in which experiment is possible. If we call the initial quantity c, and the quantity absorbed C, we have in every case yet examined the relation $C = \xi c$, just as with sensitizing agents and complement. I realize that this relation is an unusual one, and I have tried repeatedly to show that the quantity absorbed is related to the quantity initially present in a manner which would indicate an " adsorption." Within the range of possible experiment, however, I am sure that the relation is substantially linear.

2. The constant ξ varies with different lysins, and also, although to a lesser extent, with the type of cell used. For sodium taurocholate, ξ is about 0·33, and for sodium glycocholate about 0·25. But for digitonin it is about 0·7, and for saponin substantially zero. Digitonin, accordingly, shows great initial absorption, the bile salts a moderate amount, and saponin virtually none at all.

2—" Delayed " Absorption

1. *Method*—The measurement of " delayed " absorption is exceedingly laborious and difficult. The problem is to measure the quantity of lysin left " free " at any stage between commencing lysis and its completion, and it is clearly little use to throw down the unhæmolysed cells and then to titrate the supernatant fluid for free lysin, as can be done when one is measuring initial absorption, for liberated hæmoglobin, etc., may produce inhibition of hæmolytic activity. The method which I use is the following, conveniently described as a series of steps.

Step 1—A suspension of stromata* is prepared from the blood of the same animal as is used in the preparation of the suspension. In all the experiments which follow, rabbit red cells were used, but similar results can be obtained with the cells of man, the ox, or the sheep. To 20 cc of oxalated blood is added 40 cc of 1·0% NaCl (saline) ; the mixture is then centrifuged, and the supernatant fluid removed. The cells are hæmolysed by freezing and thawing. They should be frozen once only. To the hæmolysed mass is added 40 cc of saline, and the stromata are thrown down by centrifuging. They are then washed with saline until they are white, and are finally suspended in 40 cc of saline. If no stromata were lost in the process, the number of stromata per 1 cc of the stroma suspension would be ten times the number of cells per 1 cc of standard cell suspension. As a matter of fact, the yield is often surprisingly small.

* By " stromata " I mean the red cell envelopes, or " ghosts," and not the dense stromata of Rollett (see Ponder (1934)).

This suspension has now to be diluted so that the number of stromata contained is just equal to 0·4 cc of red cell suspension as regards effectiveness in reacting with lysin. One dilution of lysin, *e.g.*, 1 in 40,000 saponin, is selected; 0·8 cc is taken, 0·8 cc of saline added, and to the mixture is added 0·8 cc of standard cell suspension. The time for complete lysis is measured; suppose that it is 2·0 minutes. To 0·8 cc of the same dilution of lysin, plus 0·8 cc of saline, is added 0·8 cc of a mixture of equal parts of standard cell suspension and stroma suspension diluted 1 in 2, 1 in 3, . ., etc. The time for complete lysis in these latter systems is determined, and one of them will hæmolyse in 2·0 minutes. Suppose that the dilution of stroma suspension corresponding to this time is 1 in 10, then 0·8 cc of standard cell suspension is just equalled in effectiveness by 0·8 cc of a mixture of equal parts of standard cell suspension and stroma suspension, diluted 1 in 10. Hence 0·4 cc of the stroma suspension, diluted 1 in 10, is equal in effectiveness to 0·4 cc of cell suspension. The stroma suspension is accordingly diluted 1 in 10, and such a diluted suspension is equivalent, cubic centimetre for cubic centimetre, to a standard red cell suspension. The extent of the dilution, of course, varies with the yield of stromata.

Step 2—A time-dilution curve for the lysin, with 100% lysis as the end point, is plotted in the usual way, the systems consisting of 0·8 cc of lysin, 0·8 cc of saline, and 0·4 cc of the standard cell suspension. This is time-dilution curve A.

Step 3—A time-dilution curve is plotted for systems containing 0·8 cc of lysin in various dilutions, 0·8 cc of saline, and 0·8 cc of standard cell suspension. This is time-dilution curve B.

Step 4—One dilution of lysin is selected, and the time required for it to produce complete lysis of 0·4 cc of cell suspension is known from time-dilution curve A. A series of tubes, from four to six, each containing 0·8 cc of this dilution of lysin and 0·8 cc of saline, are prepared and placed in the water bath.

Step 5—The next step is to add to this dilution of lysin 0·4 cc of the equivalent stroma suspension (see Step 1), to allow this to interact with the lysin for an interval I, and then to add 0·4 cc of cell suspension. The intervals are so arranged as to vary from zero to time t, the time for complete lysis of 0·4 cc of the standard suspension by the particular dilution of lysin used. For instance, suppose that the dilution of lysin hæmolyses 0·4 cc of cell suspension (and therefore reacts in an equivalent manner with 0·4 cc of the equivalent stroma suspension) in $t = 2$ minutes, the experiment would be then arranged as follows. (*a*) First tube : 0·4 cc of stroma suspension added, and 0·4 cc of cell suspension added

simultaneously. (In practice, equal volumes of stroma suspension and cell suspension are mixed, and 0·8 cc of the mixture added.) Here I = 0. (*b*) Second tube : 0·4 cc of stroma suspension added, and after 0·2 minutes, 0·4 cc of cell suspension added. Here I = 0·2, or 0·1*t*. (*c*) Third tube : I = 0·4*t*, . . . Last tube : I = *t*. The time for complete lysis of the added cells is determined in each case.

Step 6—Referring to time-dilution curve B, the quantity of lysin left free in the system after any interval I can be found. By subtraction, we can then find the quantity which has been absorbed by the 0·4 cc of stroma suspension during any interval I.

2. *The Course of Delayed Absorption of Saponin by Stromata*—I shall first give the results obtained for saponin, as this lysin does not show initial absorption. Typical results are shown in Table I and in fig. 1,

TABLE I

1	2	3	4
0·1	0·0	40	1·0
0·2	0·22	34	0·90
0·4	0·44	32	0·80
0·6	0·66	29	0·73
0·9	1·0	24	0·60

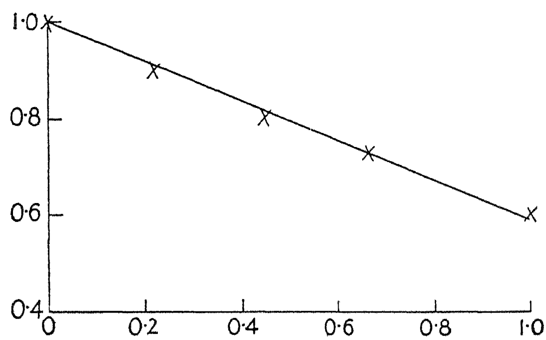

FIG. 1—" Delayed absorption " of 1 in 50,000 saponin by stromata. Ordinate, fraction of initial lysin left free ; abscissa, intervals I as fractions of *t*

the cell suspensions and the stroma suspensions being made from the blood of the rabbit. Column 1 gives the value of I in minutes. Column 2 gives I as a fraction of *t*, the time taken by the particular dilution of lysin to effect complete lysis of 0·4 cc of cell suspension. Column 3 shows the amount of lysin, in micrograms, remaining after each interval I, and column 4 gives this quantity as a fraction of the lysin initially present (40 micrograms, *i.e.*, 1 in 50,000 saponin).

Experiments of this kind are limited by practical considerations. If the dilution of lysin selected is not sufficiently great, lysis of 0·4 cc of the cell suspension is so rapid that one cannot make measurements when I is relatively small, and if the lysin dilution is very great, one cannot measure the quantity of lysin left free when I is very great, for complete lysis of the added 0·4 cc of suspension may not occur at all. The best one can do is to select a dilution of lysin for which the course of the curve which relates the amount of lysin left free to the relative interval I can be determined as completely as possible.

Reference to fig. 1 will show that the experimental points lie along a straight line. I shall discuss this linear relation directly.

3. *The Relation between the Delayed Absorption by Stromata and the Dilution of Lysin used*—Table I shows the way in which the quantity of "free" lysin present in the system decreases as delayed absorption proceeds in the case of one initial dilution of lysin. We have now to find how the quantity of free lysin present in the system at any particular stage varies with the amount of lysin initially present. The best stage to select is one which corresponds to complete lysis, when $I = t$. To 0·8 cc of a particular dilution of lysin (*e.g.*, 1 in 40,000) is added 0·8 cc of saline, and then 0·4 cc of the equivalent stroma suspension. The lysin and the stromata are allowed to interact for a time t, the same as that which would be required to produce complete hæmolysis of 0·4 cc of the cell suspension. At the end of this time 0·4 cc of suspension is

TABLE II

Lysin initially present, microgm	Lysin "absorbed" microgm
200	17
100	18
67	19
50	20
40	18
33	17
28	19

added, and the time required for its complete hæmolysis measured. The amount of free lysin present in the system at the end of the time t can then be found by referring to the proper time-dilution curve, allowance being made for the fact that the system is diluted 1·2 times by the addition of the 0·4 cc of suspension. This procedure is repeated for as many dilutions of lysin as possible ; the results are shown in Table II.

Over this quite considerable experimental range, the quantity of lysin "absorbed" by stromata at a time corresponding to complete lysis of an equivalent quantity of red cell suspension is accordingly virtually constant. The figures, of course, are not very accurate, but there is no doubt as to the general nature of the result, which is entirely different from that obtained when lysin is "absorbed" by 0·4 cc of red cell suspension instead of by 0·4 cc of an equivalent suspension of stromata (see 5 below).

Consider this result in relation to existing theory. This states that the velocity of the "fundamental" reaction between cell component and lysin is

$$dx/dt = k\,(c - x)^n \tag{1}$$

when the term which accounts for the inhibition of lysin by liberated hæmoglobin is not required, as in the systems under consideration, x being a quantity of lysin used up in combining irreversibly with the component of the membrane, c being the initial concentration of lysin, and k and n being constants. For complete lysis, x should clearly be constant, and of the same order as the concentration of lysin which produced complete lysis in infinite time (the asymptotic concentration).* Table II shows that the amount of lysin which has disappeared from the system at times corresponding to complete lysis is indeed constant, and that it is about the same as the asymptotic concentration (16 micrograms, in this case). The result is therefore wholly in accordance with theory, and I did not expect to obtain so easily this direct confirmation of an equation which has hitherto rested upon assumption only.

Turning to Table I, and looking at the results in the same light, the almost linear disappearance of lysin is easily accounted for. When the initial concentration of lysin is comparatively great, the amount of lysin which disappears as a result of combining with the cell component is virtually linear with time (expression 1), and this is the result obtained. When the initial concentration is very small, the relation between x and t is curvilinear, but I have been unable to obtain experimental results for low concentrations of lysin because of obvious technical difficulties.

If this interpretation is correct, the phenomenon of "delayed absorption" by stromata is simply the phenomenon of using up of lysin by its combining with the cell component.

* The quantity of lysin used up in infinite times as a result of its combining with stromata is not, however, the same as that used up in infinite time when the lysin combines with an equivalent quantity of red cell suspension and produces lysis of it. The reason for this is that in the lysis of the cells some of the lysin is inhibited by liberated hæmoglobin.

4. *The "Delayed" Absorption of Lysin by Red Cells*—The way in which lysin disappears from a system containing 0·4 cc of a stroma suspension equivalent to 0·4 cc of red cell suspension is not necessarily the way in which it would fall off if 0·4 cc of the cell suspension were actually undergoing hæmolysis, for in the case of the stroma suspension the lysin disappears because of "delayed absorption" only, but in the case of the red cell suspension some of the lysin is rendered inert by inhibitory substances liberated from the hæmolysing cells, and this quantity disappears in addition to that disappearing by reason of the delayed absorption. To investigate this aspect of the matter, we can modify the method in the following way. Instead of adding 0·4 cc of stroma suspension to the lysin, and then, after an interval I, adding 0·4 cc of cell suspension, we add 0·4 cc of the cell suspension to the lysin, and, after an interval I, add a second 0·4 cc of suspension. The result obtained by the two methods ought to be substantially the same, for the stroma suspension is prepared so as to be equivalent to the cell suspension. But there is this difference, that where the stroma suspension is added, the lysin which disappears must do so because of delayed absorption, whereas when the cell suspension is added in place of the stroma suspension, some of the lysin is absorbed, and some inhibited by substances liberated from the cells. The effect of this inhibition might quite well cause the relation between the amount of lysin left free and the relative interval I to be other than a straight line. Such a deviation would be very important from the standpoint of theory.

The results of this procedure are shown in Table III, drawn up like Table I (saponin 1 in 40,000).

TABLE III

1	2	3	4
0·0	0·0	50	1·0
0·1	0·17	49	0·97
0·2	0·33	45·5	0·91
0·4	0·67	40	0·78
0·6	1·0	29·5	0·59

These results are plotted in fig. 2, and again the relation is substantially linear. The similarity between fig. 1 and fig. 2 will be apparent.

5. *The Relation between the Delayed Absorption by Red Cells and the Dilution of Lysin used*—This has already been described (Ponder, 1932, *b*), and it has been shown that the quantity of lysin left free in a saponin system at the moment of the completion of lysis of a quantity of cell

suspension is not a constant quantity, but a function of the initial concentration c. Thus, instead of the type of result shown in Table II, we get the typical relation shown in Table IV.

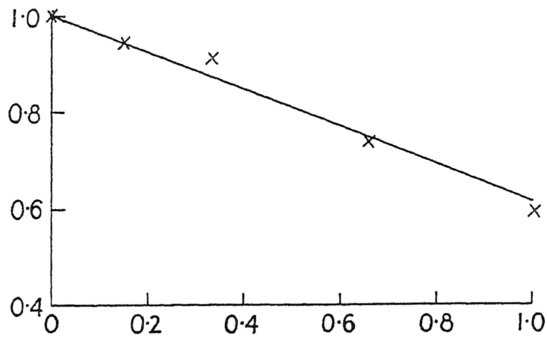

FIG. 2—" Delayed absorption " of saponin by red cells. Similar to fig. 1

TABLE IV

Lysin initially present, microgm	Lysin " absorbed " microgm
200	67
100	40
67	30
50	23
40	19

I have already pointed out that this result can be accounted for by remembering that when a quantity of red cells, as opposed to a quantity of stromata, are acted upon by a lysin such as saponin, some of the lysin is rendered inactive by the hæmoglobin liberated from the cells as they hæmolyse. Thus in the case of a system containing stromata, the quantity of lysin used up at a time corresponding to complete lysis of an equivalent amount of suspension is constant, as we have just seen (Table II), but in a system containing red cells, there has to be added to the constant amount used up a variable quantity which is rendered inert by the liberated pigment.

The assumptions already made about the inhibitory effect of the pigment liberated into such a system are that the quantity of pigment liberated is, very approximately, linear with the quantity of lysin transformed (*i.e.*, with x), and that the quantity of lysin rendered inert is proportional to the quantity of pigment present and to the initial lysin concentration. These assumptions give rise to the expression

$$dx/dt = k\{c - x(1 + Bc)\}^n \tag{2}$$

in place of expression (1), but the two expressions give the same numerical results for the time-dilution and percentage hæmolysis curves, although with different values for the velocity constant. (For a complete discussion of the assumptions and the expressions, see Ponder, 1932, *b*, and Ponder and Gordon, 1934.) The addition of the "inhibition term" also accounts for the results shown in Table IV (referred to as the "second addition effect" in Ponder, 1932, *b*) and has an important effect on the form of the derived distribution for the red cell resistances, which is much more familiar in form when the "inhibition term" is added than when it is not.

The results shown in Table III and fig. 2 can be explained in the same way as can those shown in Table I and fig. 1, for expression (1) applies to the latter systems, in which the lysin reacts with hæmoglobin-free stromata, and expression (2), which gives the same numerical results if the velocity constant is changed, to the former systems in which the lysin reacts with red cells and their liberated pigment. It is interesting to observe that the relation between the quantity of lysin left free in the system and t is substantially a linear one when the lysin reacts both with the cell component and with the liberated pigment (fig. 2), for, with the concentrations of lysin under consideration, the quantity of lysin which disappears as a result of the reaction between the lysin and the cell component is also substantially linear with t (expression (1)). This means that the quantity of lysin rendered inert as a result of a reaction with the liberated pigment is also very nearly linear both with t and with x, which is just what has been assumed, for other reasons, in giving the "inhibition term" the form Bcx. Although the assumptions underlying the treatment of the inhibitory effect of the pigment are very approximate ones, they seem to be quite adequate over the experimental range under consideration.*

6. *Absorption of Digitonin*—I shall now consider briefly the results for digitonin. This lysin differs from saponin in that it shows very considerable initial absorption. It is, however, very difficult to work with, for of all lysins digitonin shows the highest value of n, and the time-dilution curve passes upwards close to the ordinate and then turns off very suddenly towards its asymptote. The higher concentrations

* A recent monograph (Ponder, 1934) contains the statement that purified hæmoglobin is not inhibitory for saponin hæmolysis. This statement, which is erroneous, was based on the fact that one specimen of hæmoglobin, prepared by Ferry and Green's method, was found to be non-inhibitory. I have recently obtained pure samples through the kindness of Dr. Ferry and Dr. Anson, and these, as well as several pure specimens prepared in the laboratory, were inhibitory.

accordingly give such rapid lysis that it is almost impossible to use them in experiments of this kind, while the higher dilutions take so long to produce complete lysis that they are equally useless. The range of dilution over which the relation between the amount of free lysin and the relative interval can be investigated is therefore very small.

The results of a typical experiment are shown in fig. 3, in which the crosses represent points obtained by adding first stroma suspension and then red cell suspension, as in fig. 1, while the circles represent points obtained by adding first 0·4 cc of cell suspension and another 0·4 cc thereafter, as in fig. 2. The contrast between figs. 1 and 2 and fig. 3

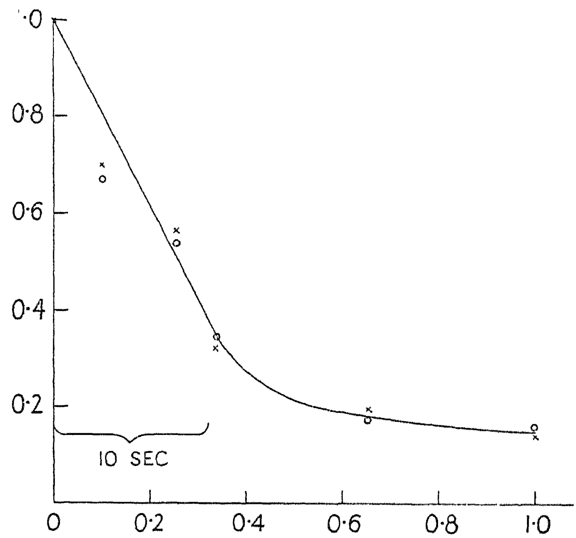

FIG. 3—" Absorption " of digitonin, initial absorption taking place within the first 10 seconds and slower " delayed absorption " thereafter. Ordinate and abscissa as in figs. 1 and 2

will be seen at once, for in the former the quantity of free lysin present in the system falls off in a linear fashion (delayed absorption), whereas in the latter there is first a very sharp fall (initial absorption), and then a slow fall like that in figs. 1 and 2. The initial absorption is complete within about 10 seconds, and it will be observed that substantially the same results are obtained irrespective of whether 0·4 cc of stroma suspension or 0·4 cc of the equivalent red cell suspension is added first to the system.

3—DISCUSSION

The results of this investigation, which can be discussed very briefly, throw considerable light on the adequacy of the theory which I have

advanced to account for what is known about the kinetics of hæmolysis in simple hæmolytic systems, and also on other theories which have been put forward from time to time.

It seems that we have now to regard hæmolytic systems as of two kinds: (*a*) those in which the system consists of cells suspended in a solution of lysin, the molecules of which react with the cell component, and (*b*) those in which the system contains cells surrounded by a region in which lysin is concentrated, this being again surrounded by a solution of more dilute lysin. The first is the sort of system met with when saponin is the lysin, and where there is no " initial absorption " ; the latter sort is met with when digitonin or the bile salts are used as lysins, in which case there is considerable " initial absorption." To distinguish between the two, I shall speak of systems which contain both " internal " and " external " lysin systems, as in the case of digitonin and the bile salts, and those which contain " external " lysin systems only, as in the case of saponin.

In those systems in which the lysin is not concentrated at the cell surfaces, the lysin and cell component appear to react according to an irreversible reaction of the *n*th order (expression (1)). Hitherto this has been an assumption, but measurements now show that a constant quantity of lysin is, in fact, used up at times corresponding to complete lysis. Further, so far as I have been able to investigate the matter, the rate at which lysin is used up is the same as that which would be predicted from the theory. The rate of utilization of the lysin, and the using up of the constant quantity at times corresponding to complete lysis, can, of course, be found only when the lysin reacts with hæmoglobin-free stromata, which do not liberate inhibitory substances.

When the lysin reacts with cells, and not with hæmoglobin-free stromata, the fundamental reaction between the cell component and the lysin seems to be the same as before, but the quantity of lysin left free at any stage appears to be less because some of it is inhibited by the liberated pigment. If we suppose that the amount so rendered inert is proportional to the quantity of liberated pigment and to the initial concentration of the lysin, the " second addition effect " is accounted for quantitatively. At the same time, the frequency distribution of red cell resistance which, in the absence of any allowance for the inhibition, is a very skew one with a long " tail," becomes a distribution which, although still skew, is of a more usual form.

In the case of those systems in which there is an " internal " lysin system surrounding the cells, and brought about as a result of " initial absorption," we have no experimental evidence as to whether the velocity

of transformation of the cell component by its reaction with the lysin is the result of an irreversible reaction of the *n*th order, or of some other reaction. But, arguing from analogy in the absence of possible experimental investigation, we can say that it is very likely that it is the concentration of free lysin in the " internal " lysin system which determines the velocity of transformation of the cell component. This point of view is strongly supported by the experimental fact that the concentration of lysin in the " internal " lysin system is a constant fraction of that in the " external " lysin system. If we consider the lysin concentration in the " internal " lysin system instead of that in the " external " lysin system, we have clearly the same confirmation of the existing theory of hæmolysis in the case of the lysins sodium taurocholate, sodium glycocholate, and digitonin as we have in the simpler case in which there is no " initial absorption." It will certainly be remarked that in the case of lysins which are " initially absorbed " there ought to be a certain equilibrium between the " external " lysin system and the " internal " one, in which the concentration of free lysin must be continually falling as lysis proceeds. What little evidence we have indicates, however, that the " internal " lysin system, once formed, is almost completely separate from the " external " system surrounding it (see Ponder and Gordon, 1934).

Quite a number of other theories which have been put forward have introduced the idea of an " absorption," or rather an " adsorption," of lysin in order to account for the results obtained. Several of these theories are reviewed by Clark (1933), and one of the most elaborate is that recently advanced by Reiner (1934), which has the advantage that the constant *n* of my equations can be made equal to unity if the amount of " adsorption " postulated by Reiner (that given by the Langmuir adsorption equation) actually occurs. The experiments described above, however, show that the amount of lysin " absorbed " does not even remotely correspond to that required by an " adsorption " equation, and that the changes in lysin concentration which occur in the systems under consideration have scarcely any relation to those which the various " adsorption " theories demand.

SUMMARY

Certain hæmolysins become rapidly concentrated at red cell surfaces, a considerable fraction of the total lysin present in the system disappearing from the fluid between the cells within 10 or 15 seconds. This rapid " initial absorption " is most marked in the case of digitonin and somewhat

less marked in the case of the bile salts ; in the case of saponin it appears to be absent.

The rapid " initial absorption " is followed by a much slower " delayed absorption " of lysin during which the concentration of free lysin in the system steadily falls.

The course of this " delayed absorption " can be closely followed in the case of saponin which shows no " initial absorption." If the saponin is added to a suspension of red cell envelopes (stromata), a constant quantity of lysin disappears from the system in such time as would be sufficient for the complete hæmolysis of that number of red cells from which the stromata were obtained. The fall in free lysin concentration is virtually linear with time and this fact, together with the fact that a constant quantity disappears, is in accordance with existing theory.

The course of the disappearance of saponin from a system containing not stromata but red cells is similar, but instead of a constant quantity disappearing, the amount which disappears is roughly a constant fraction of the amount of lysin initially introduced. The difference between this result and that immediately preceding is accounted for by the fact that the cells liberate hæmoglobin as they hæmolyse and that the liberated hæmoglobin is inhibitory.

With the lysins digitonin, sodium taurocholate, and glycocholate, the phenomena appear to be essentially of the same kind, although results cannot be obtained with the same degree of quantitative exactness as in that for saponin.

The phenomena of "initial absorption," "delayed absorption," etc., which occur in these hæmolytic systems do not seem to correspond even remotely to the " adsorption " which many investigators have postulated in connection with systems of this kind.

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548 . 22
564 . 74 *Favositella*

Phosphatic Calculi in Silurian Polyzoa

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(*Communicated by W. D. Lang, Sc.D., F.R.S.—Received August* 21, 1934)

[PLATES 12–14]

INTRODUCTION

In 1884 Etheridge and Foord published a detailed description of the Polyzoan *Favositella interpuncta* (Quenstedt) from the Wenlock Limestone of Dudley, remarking on its " mural pores," which were exceptionally large and showed a somewhat irregular distribution. The authors observe that frequently these " mural pores " have been filled with concentric layers of material, which they state to be chalcedony,* and that the " pores " are often so conspicuous that on a polished surface they are clearly visible with the aid of a hand-lens. It was the presence of these so-called pores which led the authors to assign this species to the Favositidæ, in spite of its obvious monticuliporoid appearance. At that time this was not regarded as a fundamental issue, for in this country the Monticuliporoids were still classed with the Tabulate corals.

Here the matter rested until 1911 when Bassler published his work on " The Early Palæozoic Bryozoa of the Baltic Provinces." In the course of this research Bassler had carefully examined topotype material of *F. interpuncta*, and showed conclusively that it was congeneric with those Ceramoporoid Polyzoa formerly included in the genus *Bythotrypa*—a name which had been proposed by Ulrich in 1893, and which therefore had to be suppressed in favour of *Favositella* (Etheridge and Foord, 1884).

But the immediate point of interest in Bassler's account of *F. interpuncta* is his interpretation of the pore-like structures. He at once dismisses the idea that they are infilled perforations in the zooecial walls, since they present identically the same appearance in both transverse and longitudinal sections of the zoaria. Moreover, they occur, not in the wall-tissue, but as isolated bodies in the zooecial cavities. Bassler concludes : " . . . from their position and composition it is evident that they have nothing to do with the bryozoan itself, but are

* Presumably the author had in mind " cyclic chalcedony " or *beekite*.

simply rounded siliceous bodies included in the material filling the cell cavity."

Certain anomalous features in their mode of occurrence, however, at once suggested a very different interpretation from that advanced by Bassler. In the first place, if (as he supposed) they were extraneous bodies, such as silicified ooliths, they should occur quite indiscriminately in the cells of all Polyzoa and corals found in the same deposit. But extensive investigation has shown beyond doubt that, except in instances where they are derived (see p. 304), it is only in *Favositella* and closely related genera that these spherules occur. They are completely absent from the zooecia of the Polyzoa of other families which occur in the Wenlock beds with these Ceramoporoids. It is clear, then, that the spherules are far more intimately associated with the Polyzoa in which they occur than Bassler had suspected. This, indeed, is also shown by the spherules not being confined to the sediment-filled zooecial apertures, but occurring for the most part well down in the zooecial tubes, and shut in by continuous diaphragms; nor associated with the detrital material, but embedded in clear calcite, fig. 1, Plate 12 ; fig. 5, Plate 13. Again, that the spherules were formed within the zoaria is indicated by the way in which their shape is often adapted to the form of the cell in which they occur.

An early paper by Sollas (1879) on the Silurian of Rumney was noticed after the interpretation here put forward had been worked out. In this paper, written five years before that of Etheridge and Foord, Sollas gave a full description of these spheroidal bodies (p. 501). According to him, however, they were found in the cells of *Favosites fibrosus* occurring at a horizon which he equated with the Wenlock Limestone. It is nevertheless quite clear from his figure that the bodies are the same as those noted in Wenlock Polyzoa from Dudley; and re-examination of the Rumney material has, in fact, shown that the spherule-bearing forms referred to *F. fibrosus* are actually species of *Favositella*.

Sollas noted the extraordinary resemblance which these bodies bear to minute pearls, even down to the iridescent lustre which their surfaces present. He observed : "The fact that they occur in such cells as were shut off from communication with the muddy sea-bottom, and so had become infiltrated with pure crystalline dolomite, seems to point to their formation *in situ* ; . . ." However, he reached no definite conclusion with regard to them, but said (p. 502) : "One almost feels tempted to regard the oolitic granules as 'calculi' formed during the lifetime of the *Favosites* ; and although such an explanation is not probable, it may afford us a hint as to the true explanation; for

immediately after the death of the *Favosites* a good deal of organic matter would be set free, and would pervade not only the surrounding water, but the several cells of the organism . . . and it may be just possible that the oolitic granules were formed so long as organic matter existed in the mineralizing waters, . . ."

According to Sollas the spherules are formed of concentric shells of transparent calcite. Actually they have proved to be neither calcitic, nor siliceous, as the various authors had supposed, but phosphatic.

Detailed Description

Size and Shape—In the specimens of *Favositella*, etc., which I have examined, the *spheroliths*, as they may appropriately be termed, range in diameter between 0·03 mm and 0·3 mm. Sollas, however, records a maximum diameter of 1/40 inch (approximately 0·62 mm).

They also show considerable variation in shape. In section they may be circular, oval, reniform or polygonal (see Plates 12–14). The smaller ones usually approximate to perfect spheres. The larger ones, while often spherical in the early stages of growth, have become adapted with increase in size to the form of the cell in which they occur, fig. 14, Plate 14 ; or to one another, if several have grown in close proximity, fig. 1, Plate 12. Thus the spheroliths often show flattening on one or more sides. The pressure set up during the growth of the spheroliths, when they were in contact with some resisting surface, has led in many of them to a modification of the spherical form of even the innermost shells, fig. 2, Plate 12.

Although the spheroliths are now resistant and crystalline, the deformation which their inner layers have undergone, suggests that at the time of formation they were in a gelatinous condition. This is indicated, too, by the curved lines by which they are always bounded.

One may presume that when the spheroliths were small, surface-tension preserved their spherical form, for then the internal molecular forces, tending to the production of minimum surface area, would have been in excess of the external gravitational force ; but after a certain size had been reached these forces would tend to be in equilibrium, so that the least external pressure would have deformed their spherical shape. This may explain why the larger spheroliths tend to be less spherical than the smaller ones; but it must, of course, also be remembered that the larger the spherolith the greater the probability of its being in contact with some other structure.

When there are several spheroliths in one zooecial chamber, there is a tendency for them to be small. Sometimes two or three small ones

have at first grown independently, and later have been enveloped during a subsequent deposition of phosphate, and welded into a single concretionary complex.

Mineralogical Aspects—By reflected light the spheroliths of *Favositella* from Dudley appear dark brown with a faint bluish opalescence and a distinctly resinous lustre. Many of those from Rumney, on the other hand, are colourless, showing up as conspicuous white spots on fractured or polished surfaces, and bearing a striking resemblance to minute pearls, fig. 3, Plate 12, since their opalescence is not obscured by body-colour as in the Dudley specimens. These colourless spheroliths show a pronounced iridescence.*

By transmitted light, the Dudley examples are observed to vary in colour from a deep yellowish-brown to the palest straw-yellow. In sections ground to a thickness of 0·05 mm, or less, they may appear practically colourless. Although those from Rumney are in the main colourless, the outer layers have occasionally been stained reddish-brown by the infiltration of iron-oxide, with which the limestone is richly impregnated. The uniform yellowish-brown colour of the spheroliths from Dudley can hardly be attributed to the same cause, since the matrix is far less ferruginous than that of the specimens from Rumney ; but the colour is probably due to the presence of colloidal organic material.

In thin sections the spheroliths are seen to have a fine, concentrically laminated structure with a central nucleus; fig. 2, Plate 12; fig. 9, Plate 13, are typical. Even under the highest magnification, no trace of radial structure could be detected in the component laminæ.

The spheroliths consist of a mineral with a refractive index of 1·61, so that by transmitted light they appear in relief against most of the surrounding calcite.

The explanation of the fine lamination is considered later (p. 310); but it may be mentioned here that the apparent alternation of thick and thin layers, which was noted by Sollas, does not appear to be a spacing due to Liesegang rhythm, but rather an optical effect, caused by slight variations in refractive index, and the result, no doubt, of changes in the composition of the precipitated material. This banded effect, which does not represent the ultimate lamination, is sometimes exaggerated by the

* An interference phenomenon, commonly brought about by the reflection of light at a series of superposed surfaces, the intervals between which are of the order of light wave-lengths (see p. 300).

inclusion of impurities along certain layers, indicating brief pauses in growth. An example of this is illustrated in fig. 8, Plate 13. In this case the spherolith has grown in two major stages, probably indicating an appreciable pause in deposition; but except in a few cases like this, the spheroliths were apparently formed by nearly continuous accretion.

What appear to be the ultimate layers under an $\frac{1}{8}$-inch objective, can be resolved into even finer laminæ under a $\frac{1}{12}$-inch (oil immersion) lens. The thicknesses of the ultimate layers appear to be of the order of $n\,(10^{-4})$ mm, where n is an integer less than 10. This is in agreement with the spacing required for the production of iridescence (Rayleigh, 1923).

The mineral is cryptocrystalline.* Under crossed-nicols, first-order greys are obtained, indicating low bi-refringence. On account of the spherulitic structure there is an aggregate polarization effect; that is to say, a black cross is produced with its arms at right angles and lying in the principal vibration-directions of the polarizer. This is the typical " spherulitic figure " (Morse and others, 1932).

An important factor in identifying the mineral is the specific gravity, which is comparatively high (2·95–3·0), and, apart from the other evidence, excludes it from being either calcite or chalcedony.

Some of the spheroliths were isolated from their matrix and subjected to a number of micro-chemical tests. They were found to be readily soluble in acetic and dilute hydrochloric acids. A positive reaction was obtained for phosphate, using a special elaboration of the ammonium molybdate test, involving the use of benzidine.†

The surrounding matrix of calcite gave no reaction when subjected to the same test.

The only phosphates possessing physical properties similar to those enumerated are the mineral forms of calcium carbo-phosphate. These comprise a number of definite mineral species, the more important of which include a crystalline form termed *dahllite*, and a number of colloidal and metacolloidal varieties generally grouped together under the name of *collophanite*. The latter is quite often weakly bi-refringent, as a result of the strains set up during the hardening of the gel. But, the high specific gravity of the spheroliths excludes collophanite; and it seems probable from the following comparative list of calcium carbo-phosphate

* This was confirmed by an X-ray analysis, fig. 4, Plate 12, which was kindly undertaken by Mr. H. Terrey in the Chemistry Department at University College. The continuous nature of the diffraction lines indicates the small dimensions of the constituent crystals.

† This is suited to micro-chemical work since the test depends on the formation of a deep blue compound.

minerals (compiled from Larsen and Berman, 1934) that the spheroliths consist of that form of dahllite which has the composition $2[Ca_3(PO_4)_2] . CaCO_3 . \frac{1}{2}H_2O$. This is a fibrous, and almost colourless, variety of the mineral.

Assuming that the spheroliths are composed of dahllite, it is possible to make deductions about their sub-microscopic structure. Between crossed nicols, it is found that a slow quartz wedge gives compensation

	Refractive index	Specific gravity
Collophanite	varies 1·57–1·63	varies 2·6 to 2·9
Francolite	ω 1 625	3·1
Podolite	α 1·622	3·08
	γ 1·630	
	β 1·630	
Dahllite var. a	*e* 1·631	3·08
$2[Ca_3(PO_4)_2] . CaCO_3$	ω 1·635	
Dahllite var. b	*e* 1·609	varies 2·87 to 3·05
$2[Ca_3(PO_4)_2] . CaCO_3 . \frac{1}{2}H_2O$	ω 1·620	
Dahllite var. c	*e* 1·593	varies (?) 2·97 to 3·05
$Ca_3(PO_4)_2 . CaCO_3 . H_2O$	ω 1·603	
Mineral composing spheroliths	(mean value) 1·610	between 2 95 and 3·0

when inserted radially across a section of a spherolith. Since dahllite is hexagonal and optically negative, the vibration-direction of the fast rays will coincide with the longer axes of the crystal fibres. It follows from this that the crystal fibres forming the laminæ of the spheroliths have a radial disposition, although this is not detectable even under a $\frac{1}{12}$-inch objective.

Details of Nuclei—Each spherolith almost invariably has a distinct central nucleus, the composition of which is subject to variation. In some specimens it consists of compact, red, granular material, the nature of which will be considered subsequently, in others, of finely divided, black granules. More commonly it is composed of rather flocculent material, and sometimes appears to have a cellular structure (see fig. 2, Plate 12; fig. 9, Plate 13). It is quite possible that these types represent agglomerated tissue-cells. In fact, in a few specimens the flocculent type of nucleus, when seen in section, presents shapes which strongly suggest the secondary embryos and larvæ of living Cyclostomatous Polyzoa—compare, for instance, figs. 7 and 10, Plate 13, with Borg's (1926), Plate 10, fig. 62; Plate 14, fig. 90. If degenerating embryos and aborted larvæ were present in the zooids at the time of formation of the spheroliths, they would in all probability act as centres of precipitation, and

eventually would be hermetically sealed within these calculi, in much the same way that insects are mummified in resin drops.

The nuclei vary in diameter from about 0·01 mm to 0·08 mm.

Relationship with other Structures—The spheroliths may occupy almost any position within the zoaria. Occasionally they occur in the mesopores, but more commonly they are found in the zooecial tubes, where, as has already been stressed, they are frequently shut in by continuous diaphragms and therefore quite unaccompanied by detrital material.

When, however, they occur in the younger parts of the zooecial tubes, they are not necessarily sealed and consequently are sometimes seen to be embedded in the sediment with which the open tubes became filled after the death of the colony.

In one or two instances a spherolith has grown in a cryst-like calcareous outgrowth of the zooecial wall. When the spherolith completely fills such an enclosed space, it appears as if wall-tissue had been laid down after the spherolith had formed, fig. 11, Plate 13; but the fact that in some instances the cystiform growth is much larger than the contained spherolith is opposed to this view, fig. 12, Plate 13. It suggests that these calcareous structures had an independent existence, and are really analogous to the cystiphragms found in many of the Trepostomata (see Cumings and Galloway, 1915, p. 351), and therefore preceded the formation of the concretions, which may occur within them.

There is a general tendency for the spheroliths to occur at the bottom of the zooecial chambers, either resting on a diaphragm, fig. 1, Plate 12, or on the basal lamina, fig. 13, Plate 14. In the section illustrated in fig. 15, Plate 14, all the spheroliths occur in the initial, prostrate portions of the zooecia.

Occasionally the spheroliths, in spite of their density, have not sunk to the bottom of the zooecial chamber, but occur at the top and just below a diaphragm, or suspended mid-way between two diaphragms, fig. 17, Plate 14. Movement was then prevented either by the viscosity of the medium, or more likely, by capillary effects. That they have remained in the positions in which they grew seems to point to the infiltration of the calcite *pari passu* with the disappearance of organic material in the cells.

Granular, non-detrital material sometimes occurs alongside the spheroliths and also embedded in the calcite which fills the sealed zooecial chambers. It is of two types. The first is reddish-brown and translucent, and, from its general character and from its position in the zooecia, it is clearly the replaced *brown-body* material which occurs in

numerous Palæozoic Polyzoa, and which was described by Cumings and Galloway (1915, p. 351). Occasionally small compact masses of this material form the nuclei of the spheroliths, but in general it has been completely excluded, fig. 16, Plate 14.

The second type of granular material is bluish-black and practically opaque. Its nature is uncertain. It appears to be largely iron sulphide, but this may be a replacement. Quite commonly it forms an external coating to the spheroliths, having been pushed outwards during their growth. In addition to forming nuclei, the black granules are occasionally disseminated throughout the spheroliths. In these instances the deposition of the phosphate may have been so rapid that the impurity was entrapped. In fig. 14, Plate 14, for example, one of the spheroliths is seen not only to be crowded with the black granular material but also to lack the usual concentric lamination. It probably represents a sudden gelation of carbo-phosphate, whereas the normal spheroliths are the result of relatively slow accretion.

Occurrence—The spheroliths have been found in eight or more species of Wenlockian Polyzoa, all members of the same family—the Ceramoporidæ. Up to the present these forms have not been completely investigated, but the majority can be assigned with little doubt to *Favositella*. The two common calculus-bearing species are *F. interpuncta* (Quenstedt) and *F. squammata* (Lonsdale). Spheroliths also occur abundantly in a Wenlockian *Ceramoporella.*

The spheroliths tend to be more abundant in some species than in others, but there is a wide range of individual (zoarial) variation.

In some zoaria spheroliths occur only in about 20% of the zooecial tubes, while in others they occur in practically every tube, fig. 18, Plate 14. In some of the prolific forms, as many as six may be observed between two successive diaphragms of one zooecium.

The localities from which calculus-bearing Polyzoa have been collected include Dudley, Walsall, Much Wenlock, Buildwas, Rumney (near Cardiff), and Mülde (Gotland). In the Dudley area, where they are by far the most abundant, they occur in both the Wenlock Limestone (Middle Nodular Series) and the Wenlock Shales. At Rumney they have been recorded only from the nodular limestone which was equated by Sollas with the Wenlock Limestone. Great interest attaches to the occurrence of the dahllite calculi in an incrusting Ceramoporoid (? *Favositella sp.*) from the Gotlandian of Mülde near Frojel. This form occurs in the *c* division of Lindström; that is, the Mülde-margelsten which Hede (1921, p. 87) correlated with the Lower Ludlow Shales.

Calculus-bearing forms occur in the Wenlock Shales of Buildwas, Shropshire, and in the Wenlock Limestone of the type-area, where, however, Ceramoporidæ seem to be peculiarly rare. So far no calculus-bearing forms have been found in the equivalent beds in North America (Niagaran).

The spheroliths are occasionally found in a derived condition in the rock matrix. It is only in this condition that they are known from the Aymestry Limestone. They have been observed in specimens* of the limestone from Leintwardine, View Edge, and Coverland. There are no records of Ceramoporoids from the Aymestry Limestone ; but in view of the presence there of calculi similar to those found in the ceramoporoids of the Wenlock and Gotland formations, one can only presume that insufficient search has been made. The occurrence of these isolated calculi must be attributed to the pene-contemporaneous break-up by wave-action of the zoaria in which they originated. This view is supported by the fact that the calculi occur amidst the comminuted remains of shells, corals, etc.

Conditions of Formation

Biological Considerations—We have seen that there is conclusive evidence that the spheroliths were formed within the zoaria. This being so, the concretions might be assumed to have been formed either during some phase of the life-history of the polyzoan, or during the decomposition of the organic material, or possibly even by subsequent (*post mortem*) infiltration.

The last possibility can be discounted at once for the following reason. Incoherent brown-body material (see p. 302) occurs suspended centrally in some of the zooecia of these fossil Polyzoa, indicating that calcite crystallized out concurrently with the disappearance of organic matter, fig. 16, Plate 14. From the nature of the spheroliths themselves and from the general theory of the formation of concretions of this type (*i.e.*, from solution) it is quite certain that they could not have grown in a solid medium, but developed in a liquid or gelatinous environment—in fact, before the development of the calcite, and therefore prior to the disintegration of organic material in the cells.

Occasionally spheroliths appear to interrupt the course of diaphragms, fig. 6, Plate 13, indicating a prior origin. This provides the evidence

* Collected by Miss F. E. S. Caldwell.

required to show that spheroliths were formed during the functional life of the zooids.*

At this juncture it is necessary to consider the probable sequence of events in the life-history of the zooids, some idea of which can be obtained by a study of living Polyzoa. The recent work of Borg (1933) on the Heteroporidæ is of great importance from this point of view.

It can, I think, reasonably be assumed, on the analogy of the Heteroporidæ, that the occurrence of a series of diaphragms in Palæozoic Polyzoa is evidence that the organisms underwent alternations of degeneration and regeneration. At each degeneration the polypides of recent forms break down and give rise to brown-bodies. This process is followed by a distal prolongation of the zooids and a regeneration of the polypides. Functional polypides probably only exist in the growing zone of the zoarium. In recent forms the older parts of the zooecia, often cut off by calcareous diaphragms after each degeneration, are devoid of living polypides. Borg (1933, p. 279) points out, however, that the process of growth does not consist in the superposition of new zooids on old ones, but in the distal prolongation of pre-existing zooids, and therefore the proximal portions, even when cut off by diaphragms, should not be denoted as dead (Borg, 1933, p. 305) but simply as devoid of functional polypide-structures (the inner organs of autozooids).

The cut-off portions of the zooids would presumably contain coelomic fluid, and they would intercommunicate throughout the zoarium by means of the communication-pores (see Borg, 1926, p. 201). In fact, Borg suggests (1933, p. 361) "that the zoarium should not in the first place be considered as a colony, but rather as an individual of higher order." The cut-off portions would also contain the brown-bodies formed as a result of the successive degenerations.

From the relationship which exists in the fossil forms between the brown-body material and the spheroliths, it is evident that the latter were often formed after a period of degeneration. The fact that the spheroliths sometimes show flattening against the underside of a diaphragm indicates that the concretions continued, or even began, growing after the completion of the succeeding phase of regeneration during which the diaphragm would be formed.

Where a spherolith practically fills the lumen of a zooecial tube it is evident that the functional polypide had disappeared from that part of the zooid before the growth of the concretion was complete. In

* In view of the varying use of terms relating to polyzoan structure, the usage employed throughout the present paper is that advised by Borg (1926).

a few instances, however, we have seen that the growth of calculi preceded the separation of the functional part of the zooid by a diaphragm.

Attention has already been called to the close similarity which exists between the spheroliths and pearls, and at this point it may be relevant to inquire whether it is possible that they were both formed in the same way. Pearls have been reported from a large number of invertebrates, including Polyzoa (Korschelt, 1913), but it may be definitely denied that the spheroliths have anything in common with true pearls, as far as their mode of origin and chemical composition are concerned. Molluscan pearls are formed within the mantle tissue by *epithelial secretion* round some internal product or foreign body, and consequently consist of the same substances which compose the molluscan shell, namely, crystalline calcium carbonate and conchiolin (in fine alternating layers). The composition of the spheroliths in Ceramoporidæ, on the other hand, does not correspond with that of the ectocyst—the former being phosphatic, the latter calcitic. Moreover, the varied positions which the spheroliths occupy in the zooecia preclude the possibility that they were formed by secretion within the tissues of the cyst-wall.

The spheroliths are similar both in chemical composition and in structure with the so-called gastroliths ("crab's - eyes," Huxley, 1880) which are formed in an invagination of the stomach-wall of certain Decapod crustacea by the co-precipitation of calcium carbonate and calcium phosphate with colloids (see Korschelt (1913), pp. 181–2). These calculi form reserve supplies of lime, and at each ecdysis the gastrolith is turned out of its pocket, crushed, and then redissolved by the gastric juice, so that the phosphatized lime becomes available for impregnating the new exo-skeleton. However, we have seen that the polyzoan spheroliths were formed in the absence of polypide-structures (which include the alimentary canal), so that functionally they do not in any way correspond with gastroliths.

We are driven, then, to the conclusion that the spheroliths are inorganic concretions formed in the coelomic fluid of the zooids by precipitation of phosphate around nuclei, and that they are therefore in many respects comparable with *gallstones* and *urinary calculi.*

Chemical Considerations—Apart from the structural evidence, there are certain theoretical reasons for assuming that the spheroliths were formed within the zooids. In the first place, it is feasible that restriction to the Ceramoporoids is due to their formation being controlled by some bio-chemical peculiarity of the body-fluids of these particular

forms. Speculation may now be made as to the precise nature of this bio-chemical control.

The first possibility which suggests itself is that the composition of the body-fluids was affected by the food-supply. There is evidence according to Harmer (1931, p. 135) that Polyzoa are selective in the food they digest. If these Ceramporoids were unique in their ability to digest some marine plant, rich in phosphates, one might expect an abnormally high percentage of phosphoric esters in their tissues. Although this may possibly have been a contributory factor, it is felt that it is not in itself sufficient to account for the formation of the calculi. Certainly the wide-spread occurrence of the bodies rules out the possibility of any local environmental factors being responsible for their development.

It is well known that the formation of phosphatic calculi in urine implies that the liquid is fairly strongly alkaline. The alkalinity is usually the result of a pathological state leading to ammoniacal decomposition in the urine (see Wells, p. 516).

In normal marine animals the body-fluids are probably kept at a slightly lower p_H than that of the surrounding sea-water. The p_H of the body-fluids can be assumed to vary between 7 and 8, so that in the ordinary way they would not be sufficiently alkaline to allow the precipitation of calcium ortho-phosphate. It is therefore necessary to postulate conditions which would have caused a substantial rise in the p_H of the body-fluids of the calculus-bearing Polyzoa.

The difficulty is that the body-fluids of marine invertebrates are normally so heavily buffered with proteins and inorganic substances that marked changes in their p_H value are not possible. In exceptional cases, however, the body-fluids are poorly supplied with buffers. Redfield (1933, p. 52) has recently called attention to the comparatively unbuffered condition of the coelomic fluid in the marine worm *Urechis*. Herein may lie the explanation of the restriction of the calculi to a small group of closely related species.

The suggestion tentatively put forward in this paper is that the calculus-bearing Ceramoporoids differed from all other contemporary Polyzoa in the relatively unbuffered condition of their coelomic fluid, which consequently permitted comparatively rapid changes of p_H. An appreciable rise in p_H would result in the separation of calcium phosphate if the ionic concentrations were sufficient.

It may be assumed that the coelomic-fluids of the normal Polyzoa were well buffered with proteins, etc., and, furthermore, that these fluids were practically lacking in calcium ions because all available calcium would go

to the formation of the calcareous ectocyst, particularly during degeneration of polypides.

According to the theory advanced, it is supposed that in the calculus-bearing forms the coelomic fluid lacked these proteid buffers. Carbon dioxide would be formed continuously as a result of metabolism, and, in the absence of the proteid buffers, it would tend to accumulate, and so to lower the p_H and increase the gaseous tension. To overcome this tendency it follows that some of the calcium, which normally goes in ectocyst formation, would be retained in solution in the coelomic fluid. The carbon dioxide would then be held in the form of bicarbonate ; in this way the p_H might be maintained at a fairly high level, although not much in excess of neutrality, and the CO_2-tension kept within physiologically suitable limits.

Another result of metabolism is the hydrolysis of phosphoric esters (lecithins, nucleic acid, etc.) so that ionization-products of phosphates would be present in the coelomic fluid.

On the above hypothesis, the following system of ions can be assumed to have been present in the coelomic fluid of calculus-bearing forms :—

$$\begin{array}{ll} HCO_3^- & HPO_4^{=} \\ Ca^{++} & CO_3^{=} \end{array}$$

Under ordinary conditions these would have remained static at a certain concentration. The tendency for the concentrations of the ions to increase would be balanced by outward diffusion, and the precipitation of calcium phosphate would be prevented by the comparatively low p_H (approximately that of H_2CO_3). Furthermore calcium carbonate would be kept in solution on account of the continuous formation of carbon dioxide.

From the structural evidence it has been deduced that the calculi were in all probability formed during, and immediately after, the degeneration of the polypides. At such times it is reasonable to suppose that there would be a certain amount of ammoniacal decomposition of proteid substances.* The liberated ammonia would, in the absence of heavy buffering (*ex hypothesi*), cause the p_H to rise to a sufficiently high level to allow the dissociation of HPO_4 and therefore the precipitation of calcium ortho-phosphate. At the same time the ammonia would combine

* This was the basis of *Die Eiweisstheorie* of Steinmann (1889) to account for the precipitation of calcium carbonate by plants. For a discussion of this and allied questions see Pia (1933), p. 154 *et seq.*, and (1934), pp. 42–3.

with the excess carbon dioxide, react with the calcium bicarbonate, and so precipitate the insoluble carbonate. The simultaneous formation of ammonium carbonate (an electrolyte) would facilitate the separation of the insoluble reaction-products and serve to maintain the p_H at a high level.

Whether the simple phosphate or the carbo-phosphate was precipitated in the first place would depend on their respective solubilities. Unfortunately the solubility of calcium carbo-phosphate does not appear to have been investigated.

It seems most probable that as the net result of the reactions the carbonate and the phosphate were co-precipitated,* initially, as a colloidal gel. At any rate dahllite is probably to be regarded as a solid-solution of calcium carbonate in calcium phosphate.

Lastly, account must be taken of the physico-chemical mechanism involved in the deposition of the phosphate in the form of laminated concretions. A great deal of light has been thrown on concrement formation by the work of Schade (1909). The general principles underlying the genesis of calculi in mammals under pathological conditions apply equally well to the formation of hailstones, oolites, and other spherical concretions. This has been proved by the experimental work of Schade, although geologists have been slow in accepting his results (see Bucher, 1918, p. 593 ; Schade, 1928, pp. 829–844).

Precipitation from supersaturated solutions is not alone sufficient to induce the growth of concretions, even in the presence of nuclei (Schade, 1928, p. 805, *et seq.*). Concrement-formation, according to Schade, depends firstly on the conditions being such that the substances separate intermediately in the form of droplets (" guttulate separation "), so that an emulsion is produced. This is normal for ' permanent ' colloids. In certain crystalloids it is sometimes possible, because at a certain optimum p_H an intermediate colloid-phase may separate and inhibit immediate crystallization (Schade, 1928, p. 811). The second factor in concrement-formation is the presence of surfaces or centres with adsorptive powers, on or around which the droplets will form layers. The importance of

* This is supported by an experiment carried out at the suggestion of Mr. H. Terrey. An attempt was made to reproduce as far as possible the system of ions postulated above. The concentrations were based mainly on those obtaining in the blood of living animals. 0·1 gm of calcium ortho-phosphate and 0·06 gm of calcium carbonate were shaken up in 100 cc of water. Dilute hydrochloric acid was added drop by drop until the suspended material was completely dissolved. On the gradual addition of ammonium hydrate, a bluish-white, flocculent precipitate was formed. This was found to consist of calcium phosphate and calcium carbonate, indicating that under the conditions demanded by the hypothesis, the two salts would be co-precipitated.

adsorption, as a factor in the formation of concretions, cannot be over emphasized.

That the precipitated phosphate did not form an incrustation on the sides of the zooecia lends support to the idea that at the time of the formation of the calculi the zooecial tubes were lined with healthy tissue ; because only then would there be absence of interfacial tension.*

If the precipitate is pure and the adsorbed droplets pass rapidly to the crystalline state, the resulting concretion will have a radial structure (*e.g.*, pure cholosterol gall-stones). On the other hand, if the precipitated substance remains colloidal, as in albumin gall-stones, the calculus is concentrically layered. The intermediate type is the commonest, where a colloid is precipitated along with a crystalloid, or where two crystalloids are precipitated alternately. In such types concentric lamination is combined with a radial structure, as, for instance, in the common gall-stone which consists of alternating layers of cholosterol and calcium bilirubin.

The polyzoan calculi are of a type which might be considered as intermediate between the second and third varieties. As far as their microscopic structure goes they are concentrically layered (" schalensteine " type) without a trace of radial structure, in spite of the fact that they are composed of a crystalline form of calcium carbo-phosphate. There are indications (p. 301), however, that minute crystals with a radial distribution of their optic axes are present.

The phosphate and the carbonate (or the carbo-phosphate) would have been precipitated in an emulsoid condition. They are all examples of the so-called " gelatinous salts " (see Bucher, 1918, pp. 596–7). The droplets forming the emulsion would be drawn towards readily accessible adsorptive centres. It is very probable that, should they have been present in the coelomic fluid, decomposing secondary embryos, aborted larvæ, clumps of tissue-cells, or fragments of gelatinous brown-body material, all of which would possess high adsorptive properties, would have served as nuclei for the formation of the calculi.

Surface-tension combined with de-hydration would cause the adsorbed droplets to coalesce and flatten out, and so to form a thin continuous

* The surface-tension between healthy mucus membrane and the fluid bathing it is practically nil, while between the fluid and, say, diseased tissue-cells there is considerable tension, so that fragments of diseased tissue form important adsorptive centres. It is for this reason that when the bile is supersaturated, incrustations do not form on the inner surface of the gall-bladder, but on clumps of bacteria or diseased tissue-cells (see Schade (1928), p. 818). On the other hand, in cases where the walls of the gall-duct are inflamed there is interfacial tension, and " tube casts " form.

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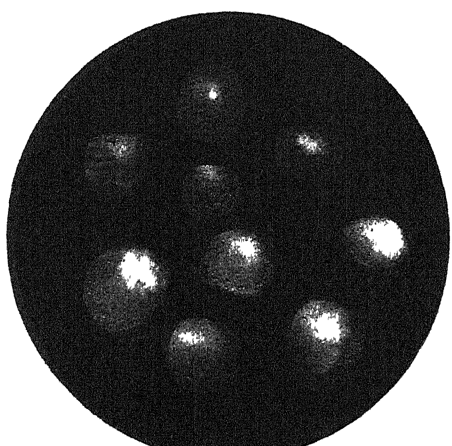

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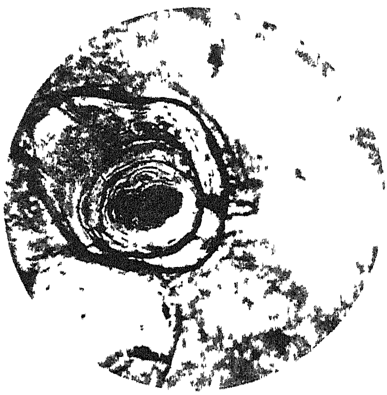

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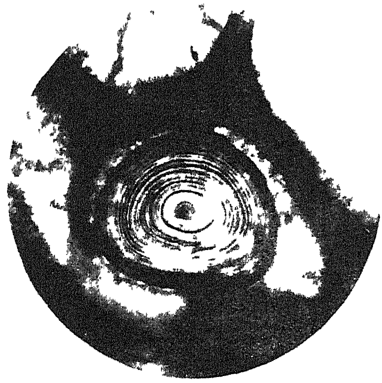

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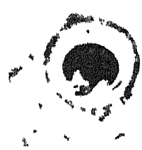

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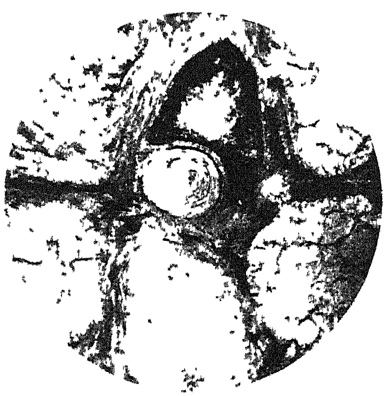

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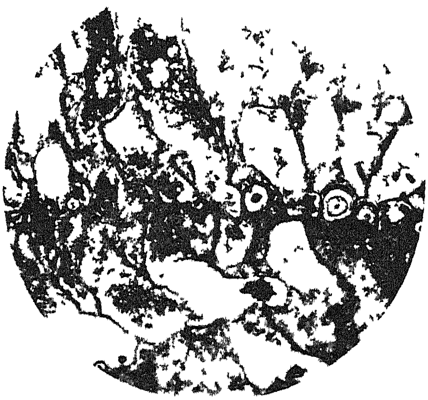

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layer over the nucleus. This process, which has been observed by Schade under experimental conditions, would be subject to continuous repetition. Each layer would adsorb colloidal material from the body fluid and this would in its turn form a fresh adsorptive layer on which guttulate separation of the phosphate would take place. According to Schade the very slightest trace of colloid in the medium is sufficient to aid concrement formation, so that this conclusion is not necessarily a contradiction of the previous assumption regarding a practically unbuffered coelomic fluid.

As each layer formed and became de-hydrated, the colloidal carbophosphate, presumably, became sub-microcrystalline by a process of polymerization. This is a process well known in many colloidal precipitates, *e.g.*, aluminium hydroxide is usually precipitated as a gel and subsequently becomes microcrystalline.

CONCLUDING REMARKS

If the assumption is correct that the occurrence or non-occurrence of phosphatic calculi in Polyzoa is dependent on the buffer-value of the coelomic fluid, it would not be in the least surprising to find calculi in isolated and quite unrelated forms. In the majority of cases such biochemical features have no phylogenetic significance whatever. This is well illustrated by the sporadic distribution of hæmoglobin in unrelated groups.

In the present case, however, whatever the pre-disposing condition may have been, it seems to have characterized practically all the later members of the Ceramoporidæ, in the British area at least, and probably over a considerably wider area. This suggests some definite correlation with inherited characters. The condition might, for instance, have been associated with the physiology of ectocyst-formation, since the ectocyst is known to be of a peculiar composition in Ceramoporidæ.

Unless there has been more extensive homeomorphy than anyone would at present be prepared to admit, the calculus-bearing forms belong to genera (*e.g.*, *Favositella*, *Ceramoporella*) which were already well differentiated in Ordovician times; and yet so far as is known the calculi were only developed in the Upper Silurian species of these genera. This raises the question whether the Ceramoporidæ in the North European area were affected by some environmental change in Wenlockian times, or whether we are to see at work, here, a definite trend, such as a progressive loss of buffer-substances in the coelomic fluid, which led to the extinction of this family at the end of the Silurian. If

the latter is the correct explanation, the formation of calculi must be regarded as the pathological entail of a korontic condition of the latest Ceramoporidæ.

I wish to thank the Department of Scientific and Industrial Research for a grant which has enabled me to undertake the investigation, and also Professor W. B. R. King under whose direction the work has been carried out. I am much indebted to Dr. W. D. Lang, F.R.S., for his helpful criticism of the paper, and for granting me facilities at the British Museum (Natural History). My best thanks are also due to Dr. H. D. Thomas for reading through the typescript, to Dr. M. Macgreggor, Mr. H. Terrey, Mr. N. H. Howes, Mr. Campbell Smith, Miss Lang, and Dr. Janet Matthews for help in various ways, and to Dr. North, of the National Museum of Wales, to Mr. A. G. Brighton, of the Sedgwick Museum, and to Dr. Folke Borg, of Uppsala, for the loan of material from the collections under their care. I should like to acknowledge assistance with the photomicrography from Mr. P. A. Johnson, technical assistant in the Geology Department at University College, London.

Summary

The pearl-like bodies (spheroliths) found in the zooecia of certain Ceramoporoid Polyzoa (*Favositella*, *Ceramoporella*, etc.) occurring in the Wenlock formation consist of calcium carbo-phosphate (dahllite).

They were apparently formed within the zoarium before the final disintegration of the zooids which occupied the zooecia.

The probability is that they were formed in the coelomic fluid of the cut-off distal portions of the zooids after periods of polypide-degeneration.

Their restriction to a few closely related genera is attributed to a biochemical control effected by the coelomic-fluids of these particular forms.

Two of the operative factors in the precipitation of the phosphate are believed to have been a lack of buffering in the fluids and the consequent rise in p_H during the degeneration of polypide-structures.

The spheroliths are thus seen to be of the nature of calculi and may be compared to such pathological structures as human gall-stones and bladder-stones.

Their mode of formation can be adduced on the basis of the experimental work of Schade on the production of concretions.

It is suggested that the postulated loss of buffers in the coelomic fluid may have been the result of a definite trend, when the formation of

calculi would have been a sign of the gerontic condition of the Upper Silurian Ceramoporidæ.

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Description of Plates

Plate 12

Fig. 1—*Favositella interpuncta* (Quenstedt). Longitudinal section. × 60. "Wenlock" Limestone, Dudley. Brit. Mus. D33702. Shows typical position of spheroliths within a zooecium.

Fig. 2—Section of spherolith showing details of structure. Taken from transverse section of *Favositella* sp. × 140. "Wenlock" Limestone, Dudley. Brit. Mus. D33685.

Fig. 3—Group of selected spheroliths. × 50 (approx.). Separated from specimen of *Favositella* sp. "Wenlock" Limestone, Ty Mawr Lane, Rumney. Nat. Mus. Wales, G658.4.

Fig. 4—X-ray pattern given by sample of powdered spheroliths (dahllite). Photograph taken through the courtesy of Mr. H. Terrey, University College, London.

PLATE 13

FIG. 5—*Favositella interpuncta* (Quenstedt). Longitudinal section. × 30. "Wenlock Shales," Dudley. Brit. Mus. D33696. Shows the occurrence of spheroliths sealed in by a diaphragm and unaccompanied by extraneous material.

FIG. 6—*Favositella interpuncta* (Quenstedt). Longitudinal section. × 75. "Wenlock" Limestone, Dudley. Brit. Mus. D33689. A spherolith is seen to interrupt the course of a diaphragm indicating the priority of the former.

FIG. 7—Nucleus of spherolith, bearing resemblance to section of polyzoan "secondary embryo." See Borg, 1926, pl. 14, fig. 90. Taken from section of *F. interpuncta* (Quen.). × 420. "Wenlock" Limestone, Dudley. Brit. Mus. D33702.

FIG. 8—Spherolith with apparent evidence of marked pauses in growth. Taken from section of *Favositella* sp. × 130. "Wenlock" Limestone, Dudley. Brit. Mus. D33686.

FIG. 9—Spherolith with nucleus composed of "cellular" material. Taken from section of *Ceramoporella* sp. × 130. "Wenlock" Limestone (Middle Nodular Series), Wren's Nest, Dudley. Brit. Mus. D33925.

FIG. 10—Spherolith with nucleus resembling section of larval polyzoan. Compare Borg, 1926, pl. 10, fig. 62. Taken from transverse section of *F. cf. interpuncta.* × 110. "Wenlock" Limestone, Dudley. Brit. Mus. D33729.

FIG. 11—*Favositella cf. interpuncta* (Quen.). Transverse section. × 55. "Wenlock" Limestone, Dudley. Brit. Mus. D33729. Shows spherolith completely filling "cystiphragmic" inflation of the zooecial wall.

FIG. 12—"Cystiphragmic" structure containing a partly decomposed spherolith. Taken from section of *Favositella* sp. × 75. "Wenlock" Limestone (?), Dudley. Brit. Mus. D33721.

PLATE 14

FIG. 13—*Favositella* sp. Longitudinal section. × 60. "Wenlock" Limestone, Dudley. Brit. Mus. D33685. Spheroliths at bottom of zooecia and resting on basal lamina.

FIG. 14—*Favositella cf. interpuncta.* Transverse section. × 55. "Wenlock" Limestone, Dudley. Brit. Mus. D33729. One of the spheroliths (on right) is crowded with black granules.

FIG. 15—*Ceramoporella* sp. Longitudinal section. × 24. "Wenlock" Limestone, Dudley. Sedgwick Museum A5899d. Spheroliths are seen to occur at the base of the zooecial tubes which form the upper layer of the zoarium.

FIG. 16—Reniform spherolith which appears to have excluded *brown body* material during growth. Taken from section of *Favositella* sp. × 150. "Wenlock" Limestone, Dudley. Brit. Mus. D33685.

FIG. 17—*Favositella* sp. Longitudinal section. × 30. "Wenlock" Limestone, Dudley. Brit. Mus. D33685. Shows the occurrence of spheroliths at both top and bottom of zooecial chambers.

FIG. 18—*Ceramoporella* sp. Transverse section. × 24. "Wenlock" Limestone (Middle Nodular Series), Wren's Nest, Dudley. Brit. Mus. D33926.

(Abstract)

612.014.481.1

The Physical Basis of the Biological Effects of High Voltage Radiations

By W. V. MAYNEORD, D.Sc., F.Inst.P.

(*Communicated by H. Hartridge, F.R.S.—Received May* 26, 1934)

The possible importance of the range of the secondary electrons produced in living materials by high voltage X-rays and gamma rays is discussed. Using the Klein-Nishina formulæ, the total path of all the electrons set free per unit energy absorption is calculated and shown to rise rapidly in the gamma ray region of wave-lengths.

Supposing one or more electron passages suffice to cause a biological change in a cell, the survival curves of a mass of cells irradiated with different wave-lengths are calculated. The differences in these survival curves according to the energy absorption required to cause the biological changes are discussed.

(*The full paper is published in* ' *Proc. Roy. Soc.*,' *A*, *vol.* 146, *p.* 867 (1934).)

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Experiments on the Development *in vitro* of the Avian Knee-Joint

By HONOR B. FELL, Royal Society Messel Research Fellow, and R. G. CANTI, Strangeways Research Laboratory, Cambridge, and St. Bartholomew's Hospital, London

(*Communicated by R. Robison, F.R.S.—Received August* 8, 1934)

[PLATES 15–18.]

1—INTRODUCTION

The subject of joint-formation, about which there has been much speculation and conflicting hypothesis, involves two main problems:

(1) The question whether the characteristic shape of the articular surfaces is due to extrinsic or intrinsic factors.

(2) The question how two opposing bone rudiments developing from a common block of mesoderm are able to separate and form two movable, independent units, instead of developing in continuity.

The first problem appears to have been finally settled by Murray (1926). This author, by grafting fragments of the early embryonic limb-rudiment of the fowl on to the chorio-allantoic membranes, was able to show that a rudimentary bone with its characteristic articular surfaces can develop in the complete absence of the normally contiguous structures. He concludes from this that the general form of the bone is determined by intrinsic factors, although mechanical influences produce minor sculpturing. This conclusion has been confirmed by Fell and Robison (1929).

The second problem, namely, the method of separation of adjacent articular surfaces, is the subject of the present communication.

It is possible to imagine various factors which might be wholly or partially responsible for joint-formation in normal development:—

(1) Murray (1926) and Warren (1934) have demonstrated that the avian limb-rudiment is a mosaic system, and the separation of the articular surfaces might be an expression of the already existing mosaic. For example, a thin layer of non-chondrogenic tissue might extend transversely across the knee-joint region, so that chondrogenesis would

stop automatically on either side of the layer which would thus provide a " breaking-plane." Alternatively, the joint might be formed by a streaming of cells away from the articular region towards the chondrogenic centres, or by some other co-ordinated form of active mass movement of the articular tissue.

(2) The development of the joint might be controlled by the development and activity of the muscles, since in the fowl, early myoblasts appear in the limb-rudiment at the stage when the first indication of the future knee-joint becomes distinguishable.

(3) The nerve and vascular supply might be concerned.

(4) Joint-formation might in some way be the mechanical result of differential growth, *e.g.*, the expansion of adjacent chondrification centres might produce a joint by compressing the intervening tissue (Carey, 1922).

The various theories concerning joint-formation formulated by previous workers have been admirably reviewed and discussed by Murray and Selby (1930) and need not therefore be considered at length in this communication.

In a previous paper Fell and Robison (1929) showed that the femur rudiment from $5\frac{1}{2}$–6 day fowl embryos underwent considerable anatomical and histological development when removed from the limb and cultivated *in vitro* by the watch-glass method. The same technique was particularly suitable for an experimental study of joint-formation (*a*) because of the complete isolation of the skeletal rudiment which it involves, (*b*) because of the ease with which the rudiments can be manipulated and (*c*) because the explants can be subjected to direct microscopic observation at any stage of the experiment, which greatly helps the interpretation of results.

2—Technique

Histology—With the exception of a few explants fixed in Carnoy's fluid, all the material was fixed in Zenker's solution with 3–4% glacial acetic acid for 1–3 hours, after which it was washed, dehydrated, cleared in clear wood oil and embedded in paraffin wax. Most of the serial sections were stained with safranin and picro-indigo-carmine, but some were stained with thionin or with Delafield's hæmatoxylin and eosin.

Tissue Culture—All the explants were grown by the watch-glass method (Fell and Robison, 1929). In this technique the culture vessel is a watch-glass enclosed in a petri dish carpeted with a layer of wet cotton-wool, in which a large round hole has been cut to allow transillumination of the

explants for microscopic study. The watch-glass contains a clot composed of 4 drops of fowl embryo extract and 4 drops of fowl blood plasma. For the present experiments the extract was made with Pannett and Compton's saline from the minced tissue of a 10–11 day embryo.

The explants, which were cultivated on the surface of the clot, were removed from the old medium every 48 hours, washed in saline and transferred to a fresh clot in another culture vessel. For the experiments described in Parts III–VI great care was taken to maintain the original orientation of the explants when transferring them to another watch-glass.

Before the skeletal rudiments were dissected for explantation, a projection drawing was made of one limb-bud from each embryo used, so that the length of the original bud could be measured and its degree of development ascertained by referring to sections of a normal limb-bud of the same length.

Cinematography—The general technique employed is that described by one of us (Canti, 1928).

The photographs were taken at intervals of $2\frac{1}{2}$ or 5 minutes, and yielded on projection a speeding up of approximately 2500 or 5000 times respectively.

The objectives used were a 3·5 or a 5 inch photographic lens placed near the objective and distant from the film.

The lighting was oblique from below, and was arranged in such a way that the main beam just avoided entering the objective. A brilliant dark-ground illumination effect was thus obtained.

3—The Early Histological Development of the Knee-joint *in vivo*

Object of Investigation—(*a*) To study the normal histogenesis of the knee-joint during the first half of embryonic life in order to provide a basis for experimental work; (*b*) to determine whether the tissue at the site of the future articulation is chondrogenic in normal development.

Material—The material consisted of serial sections of the limb-skeletons of thirty embryos ranging in age from 4 to 10 days.

Results—The rudiment of the limb-skeleton first appears when the leg-bud is about 1 mm in length and is represented by a diffuse condensation of mesoderm in the proximal part of the bud. The cells of this condensed region are not arranged in an orderly fashion as in pre-

cartilage and the mass merges imperceptibly with the surrounding loose mesenchyme.

In a 1·6 mm bud the mass of condensed mesoderm has become Y-shaped. The tail of the Y, which is proximal and is destined to form the femur and pelvis, is very short and thick and is composed of fairly compactly arranged cells, whilst the two arms, which eventually give rise to the tibia and fibula, are rather less differentiated. The margin of the Y-shaped mass is everywhere extremely diffuse and indefinite. Distally the tibia and fibula arms are separated by rather loose vascular mesoderm, but proximally this tissue gradually merges with an elongated patch of more densely packed cells lying in the femur region, and it is thus impossible to determine exactly where the bifurcation begins. This elongated patch is very conspicuous in the living bud since it appears dark when viewed with transmitted light owing to the presence of large numbers of opaque, degenerate cells. The significance of this partly degenerate area is not known, but, as will be seen later, it formed a very useful landmark in experimental work and for convenience will be henceforth referred to as the "opaque patch."

The Y-shaped skeletal rudiment elongates rapidly. In a 2·3 mm bud, fig. 1, Plate 15, the femoral portion consists of well-formed precartilage whilst the two arms representing the tibia and fibula are composed of rather less developed precartilage. The opaque patch which enlarges slightly during the earlier stages in the development of the limb is very distinct in the 2·3 mm bud, but has begun to diminish in size. Early myoblasts have now differentiated in the surrounding mesenchyme, and the tarsal and metatarsal regions are faintly distinguishable as very diffuse condensations of mesoderm.

Cartilage matrix giving the typical metachromatic coloration with thionin, is first seen in a bud about 2·8 mm in length, fig. 2, Plate 15. The rudiments of the tibia and fibula are still directly continuous with that of the femur, but in the middle third of each of the three rudiments the cells have begun to enlarge and have become enclosed by delicate walls of chondroid matrix, whilst the knee-joint region still consists of a dense mass of undifferentiated mesoderm. Chondrification is always slightly more advanced in the fibula than in the tibia. Between the chondrifying tibia and fibula is an area of indifferent, loose mesoderm containing blood vessels and continuous on either side with the early cartilage, and proximally with the opaque patch which has decreased in size still further.

Very shortly after this stage, in a 3 mm bud, the first sign of the future knee-joint appears. In longitudinal section the articular boundary of

each bone rudiment becomes faintly indicated in the knee-joint region by the flattening of the terminal mesoderm cells and their orientation along curves which are roughly parallel with the future articular surfaces. There is still, however, direct continuity between the densely cellular ends of the three rudiments. Simultaneously a rather vaguely defined perichondrium of flattened cells is formed over each shaft and this merges inwardly with the young cartilage and outwardly with the surrounding mesenchyme. The opaque patch has become very much smaller and more definite, and now lies in the notch formed by the condyles and the proximal ends of the tibia and fibula. At this stage it contains few degenerate cells and its opacity is due to the density of the tissue.

The curves along which the flattened terminal cells are orientated rapidly become more pronounced and are very distinct in a 4·3 mm bud, fig. 3, Plate 15. By this stage cartilage matrix is comparatively abundant in the middle-third of the femur, tibia, and fibula, and chondrification has spread into the densely cellular tissue at the extreme ends of the three rudiments. In thionin-stained preparations it is interesting to note that cartilage matrix of a very young and immature type extends right across the line of the joint, so that the articular surface of the femur is still united to the articular surfaces of the tibia and fibula. The opaque patch occupying the proximal part of the space between the tibia and fibula has become roughly T-shaped, the broad top of the T merging on either side with the dense tissue across the joint region, whilst the narrow tail of the T, which is less dark in appearance, is continuous with the loose vascular tissue separating the more distal parts of the tibia and fibula.

About 24 hours later—that is to say, in a 6-day bud, fig. 4, Plate 15—cartilage is well developed throughout the epiphysial region, except in the highly cellular mesoderm of the joint region where chondrification is still at an early stage. This dense chondrifying mesoderm of the joint shows signs of transverse division and has become differentiated into a thick layer of highly cellular early cartilage covering, the articular surface of the femur and similar layers covering the articular surfaces of the tibia and fibula. The narrow space between the articular surfaces of the femur and of the tibia and fibula is occupied by rather loose tissue in which matrix staining metachromatically with thionin is still visible. The rudiments of the joint capsule and ligaments are now distinguishable in section as bands of elongated cells. The patella is not yet chondrified but consists of a mass of rather loose, vascular mesoderm bounded externally by a well-defined fibroblastic membrane and internally by the perichondrium of the bone rudiments.

Between the 8th and 10th day of incubation, figs. 5 and 6, Plate 15, the loose tissue connecting the now sharply defined articular surfaces becomes increasingly scanty and finally completely disappears. The articular surfaces at the 10th day are covered by a thick layer of highly cellular cartilage with comparatively little chondroid matrix; this layer is fairly sharply defined from the rest of the epiphysis in which the intercellular material is plentiful. The capsule and ligaments are well developed, although the patella is not yet chondrified. The development of the knee-joint is now complete in all essential features.

Conclusion—Joint-formation is not caused by the presence of a layer of non-chondrogenic tissue across the site of the future articulation.

4—The Development of the Knee-joint *in vitro*

Object of Experiments—To determine (*a*) whether the knee-joint can develop in the absence of muscular movement, and of a nerve and blood supply, and (*b*) whether, if the joint can develop under the above conditions, it is formed by the streaming of cells away from the joint region.

Material and Methods—The great majority of the explants were obtained from 4-day embryos at a stage when the skeletal rudiment consists of a Y-shaped mass of mesoderm in which cartilage is not yet present and there is no sign of the future knee-joint, but a few were taken from both slightly more advanced and slightly younger embryos. The ectoderm and nearly all the myoblastic sheath were dissected from the skeletal rudiment which was then explanted.

Observations were made on ninety-nine explants, twenty-seven of which were controls in experiments described in the succeeding sections.

Three of the cultures were successfully filmed. In these cases the opposite limb-bud from the same embryo was fixed and sectioned at the beginning of the culture period as a control and the explanted rudiment was fixed and sectioned after 6–8 days' cultivation.

For all the other cultures, a projection drawing was made of one limb-bud from each embryo used, before the skeletal rudiment was removed.

Eight of the explants were drawn at intervals of a few hours with the aid of a camera lucida, and were fixed after short culture periods ranging from 5–48 hours. Twenty-seven explants were fixed and sectioned after 4 days *in vitro*, and were drawn immediately after dissection and at intervals of 24 hours afterwards, whilst the remainder were fixed and sectioned after 17 days and were drawn immediately after dissection and every 48 hours throughout the culture period.

Results—It was found that the early stages of joint-formation take place very readily *in vitro* and can be beautifully seen in the living culture and in the cinema films.

At the beginning of the culture period the explants consist of an oblong block of tissue, fig. 28 (*a*); fig. 7 (*a*), Plate 16, in which the opaque patch described in the preceding section is conspicuous.

In the living culture it is seen that during the first 12 hours' growth the opaque patch enlarges very rapidly until it is about three times its original size, fig. 7 (*b*), Plate 16, and forms an oblong area of which the

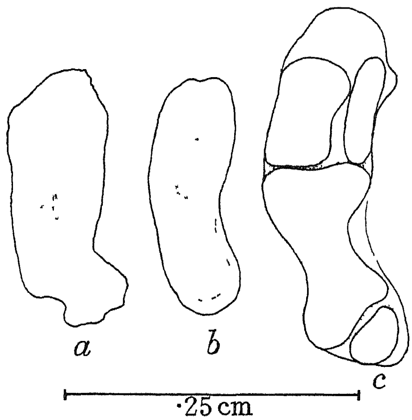


FIG. 28—Camera lucida drawings of a living explanted blastema from a 2·18 mm bud. (*a*) Immediately after explantation. (*b*) After 24 hours' cultivation; note the T-shape assumed by the opaque patch. (*c*) After 4 days' cultivation; a section of this explant is shown in fig. 8, Plate 16. *Note*—All the text-figures are drawn to the scale shown on this drawing.

distal half extends between the tibia and fibula rudiments. This enlargement of the opaque patch, particularly of the distal portion, is considerably exaggerated in the explants as compared with the enlargement which takes place in normal development. As in the normal bud the opacity is mainly due to the presence of numerous degenerate cells. Explants fixed and sectioned at intervals of a few hours show that during the first 12 hours' cultivation the originally diffuse rods of early precartilage extend and become much more definite.

Having reached a maximum the dark area rapidly diminishes in size and at the same time alters its form. As in the normal limb it becomes T-shaped, fig. 28 (*b*); fig. 7 (*c*), Plate 16; the top of the T marking the knee-joint and the tail marking the division between the tibia and fibula. Analysis of the films suggests that this change in size and shape is caused by the progressive enlargement, both in length and diameter, of the three

chondrifying regions of the femur, tibia, and fibula respectively. As *in vivo*, chondrogenesis begins in the middle third of each of the three rudiments and spreads towards the epiphysial ends. As a result, more and more of the opaque patch becomes transformed into hyaline, translucent cartilage, so that the patch finally becomes restricted to the T-shape described above. This appearance is clearly seen at the end of the first day's cultivation.

Sections of an explant at this stage show a curved arrangement of the cells near the future articular boundaries of the three cartilages exactly like that seen *in vivo*. The femur, however, is still directly continuous with the tibia and fibula at the site of the knee-joint. Around the shafts of the femur, tibia, and fibula the surrounding mesenchyme cells have become slightly flattened over the surface of the cartilage to form a rather indistinct perichondrium. The degenerate cells have almost disappeared from the mesoderm of the T-shaped area as in the normal limb-bud at a similar stage, and its opacity now appears to be due to the density of the highly cellular tissue of which it is composed, contrasted with the translucence of the surrounding cartilage.

At first the cross-bar of the T does not extend right across the knee-joint. As the three cartilages continue to develop, however, the dark line spreads completely across the joint. At the same time the rudiments of the femur, tibia, and fibula become more and more defined. Originally the tibia is only slightly larger in diameter than the fibula, but as development proceeds the tibia, as in normal development, increases in diameter more rapidly than the fibula. One or more tarsal elements are often distinguishable, but the metatarsus, when present, is usually represented only by a shapeless nodule of cartilage. The femur gradually assumes a comparatively normal shape, fig. 28 (*c*); fig. 7 (*d*), Plate 16, with condyles at the distal end and head and trochanter at the proximal end. Part of the pelvis is also almost invariably present and becomes separated from the femur rudiment by a dark line formed in a somewhat similar way to the line marking the knee-joint.

A careful study of the cinema films afforded no evidence of streaming of cells away from the joint region at any stage of development. Had such streaming occurred it would almost certainly have been demonstrated in the " speeded up " films which show cell movements very clearly.

The articular line of all the joints is most marked by about the 4th day of cultivation and the explants when being subcultivated at this stage have to be detached from the plasma clot with care, otherwise they are apt to be broken at the knee-joint. Sections, fig. 8, Plate 16, show a dis-

tinct division between the articular surfaces of the femur and of the tibia and fibula. A fairly dense layer of cells covers the articular surfaces, but the narrow intervening space usually contains very loose connective tissue. The knee-joint does not develop equally well in all explants, and in some the transverse division does not extend completely across the joint so that the tibia and fibula are united with the femur at the extreme margin.

The densely cellular layer covering the articular surfaces in the explants is not so pronounced as in the normal limb, fig. 5, Plate 15. In later development *in vitro* it completely disappears, and becomes transformed into cartilage indistinguishable from the rest of the epiphysial region.

After 6 days' cultivation *in vitro*, further joint differentiation ceases and actual fusion of the bone rudiments takes place. This is shown in the living culture by the fact that the dark line across the knee-joint becomes less and less distinct and the explant becomes increasingly rigid to handle during subcultivation. There is no suggestion of the formation of a joint cavity and sections of an explant cultivated for 17 days show direct continuity between the three cartilages at their points of contact, fig. 9, Plate 16.

The cartilaginous parts of the explants are always enclosed by loose connective tissue containing capillary spaces and occasional scattered myoblasts. The myoblasts are more numerous during the early stages of cultivation, but their distribution is always more or less chaotic and they do not exert any mechanical action on the joints which are completely immobilized on the clot.

Conclusions—(1) The earlier stages of joint-formation can proceed in the complete absence of muscular action, and of a nerve and blood supply.

(2) There is no evidence that the joint is formed by streaming movements of the cells.

(3) The joint cannot persist under the conditions of life *in vitro*.

5—The Localization of the Knee-joint

Object of Experiments—To define the site of the knee-joint at different stages of development, with reference to the opaque patch.

Material and Methods—Fragments of skeletal rudiments from limb-buds ranging in length from 1·38–3·2 mm were cultivated *in vitro* for 4 days, at the end of which time the explants were fixed and sectioned.

Camera lucida drawings of each explant were made at the beginning of the experiment and at daily intervals throughout the culture period.

Five groups of experiments were made:—

Group a—Six cultures; lengths of original buds in mm: 1·74, 1·83, 2·26, 2·96, 3·18, 3·22. A cut was made just distal to, and in one case across the opaque patch. The distal part of the operated rudiment was rejected and the entire skeletal rudiment from the opposite leg-bud of the same chick was cultivated in the same watch-glass as a control.

Group b—Seven cultures; lengths of original buds in mm: 1·48, 1·74, 2·26, 2·52, 2·7, 2·74, 2·87. A cut was made across the distal part of the opaque patch, and in one culture distal to the opaque patch. Both parts of the operated rudiment and the entire rudiment from the opposite leg-bud of the same chick were cultivated in the same watch-glass.

Group c—Six cultures; lengths of original buds in mm: 2·18, 2·31, 2·31, 2·31, 2·91, 3·05. One skeletal rudiment from each embryo was cut into three parts, the middle part being the smallest and containing all or most of the opaque patch. All three fragments and the entire rudiment from the opposite side were cultivated in the same watch-glass.

Group d—Four cultures; lengths of original buds in mm: 1·78, 1·78, 1·78, 2·39. A cut was made just proximal to the opaque patch. Both fragments together with the entire rudiment from the opposite leg-bud were cultivated in the same watch-glass.

Group e—Seven cultures; lengths of original buds in mm: 1·35, 1·96, 1·96, 2·09, 2·31, 2·35, 2·48. The rudiment from one leg-bud was cut slightly proximal to the opaque patch, the proximal fragment being fixed and sectioned, whilst the opposite rudiment from the same embryo was cut slightly distal to the opaque patch, the distal part being fixed and sectioned.

Results—1. *Proximal Fragments*—The thirteen explants in which the cut had been made just distal to the opaque patch (five from Group *a*, one from Group *b* and seven from Group *e*) all contained a complete femur and part of the pelvis.

In two of these explants two independent nodules of cartilage of unequal size, representing the proximal ends of the tibia and fibula, were associated with the condyles, from which they were clearly separated by layers of flattened cells as in the knee-joints of the control, unoperated explants. One explant showed a fairly large nodule fused laterally with one condyle and in six others there was a single well-formed nodule,

completely independent of the femur, and situated distal to the condyles. One of these independent nodules seemed to represent the fused and disorganized ends of the tibia and fibula and formed a narrow strip extending across both condyles. Isolated nodules were absent in two of the explants and in two others one or more small, very early chondrogenic areas lay in the tissue covering the end of the femur. These very small areas probably had no anatomical significance, but were the result of the disorganization and delayed differentiation caused during the early stages of cultivation by the diffuse amœboid outgrowth of the chondrogenic tissue from the cut end of the fragment.

Four of the seven fragments which were cut across the distal part of the opaque patch (one from Group *a* and six from Group *b*) developed no independent nodules distal to the femur, and two showed minute, poorly developed areas like those described above. In another specimen, from a 1·74 mm bud from Group *b*, the femur lacked one condyle and part of the other; the missing parts were associated with the tibia and fibula of the proximal fragment grown in the same watch-glass.

FIG. 29—Camera lucida drawings of the living proximal fragment of a blastema from a 2·48 mm bud. (*a*) Immediately after explantation. (*b*) After 24 hours' cultivation, showing the T-shape assumed by the opaque patch. (*c*) After 48 hours' cultivation showing the formation of a knee-joint between the femur and the proximal fragment of the tibia and fibula. A section of this explant is shown in fig. 10, Plate 16.

In order to interpret correctly the appearances seen in the histological sections, it was necessary to make a very careful study of the series of camera lucida drawings made during the life of the cultures.

One of the two specimens in which two independent nodules were associated with the condyles, fig. 29; fig. 10, Plate 16, showed all the usual stages of joint-formation during life. The opaque patch became constricted into a T-shape, and the top of the T then extended laterally so that the two nodules became separated from the femur. In the other specimen the process of joint-formation was somewhat obscured and could only be followed on one side.

An interesting phenomenon was brought to light by examining the drawings of those explants in which one independent, fairly large mass of cartilage occurred distal to one condyle. In these specimens, fig. 30, the opaque patch became T-shaped during the first 2 days' growth, but one arm and the upright of the T gradually became much more pronounced than the other arm which never extended to the margin of the joint region, rapidly became less distinct and by the third day had completely disappeared. The other well-marked arm extended laterally in the usual way and cut off a nodule which, to judge by its comparatively

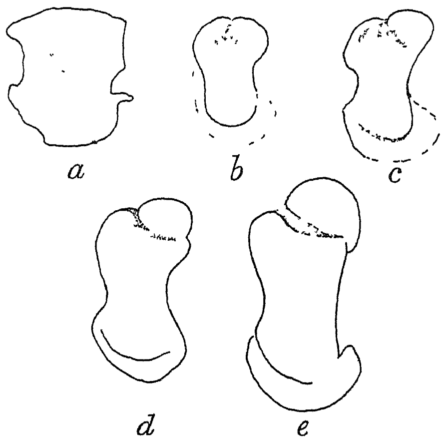


FIG. 30—Camera lucida drawings of the living proximal fragment of a blastema from a 1·96 mm bud. (*a*) Immediately after explantation. (*b*) After 24 hours' cultivation. (*c*) After 48 hours' cultivation; the opaque patch has become T-shaped. (*d*) After 3 days' cultivation; the left arm of the T-shaped opaque patch is beginning to disappear. (*e*) After 4 days' cultivation; the left arm of the T has disappeared with the result that an abnormally large condyle has been formed on that side; the right arm of the T has persisted and a large independent nodule, probably the proximal end of the tibia, has become separated from the femur. A section of this explant is shown in fig. 11, Plate 16.

large size, usually represented the end of the tibia. Owing to the early abortion of the transverse division on the opposite side, one condyle of the femur was abnormally large. This enlargement was not caused, strictly speaking, by a fusion of the femur with the end of the fibula, as the fibula rudiment was never separated from the femur, but was rather an incorporation with the substance of the condyle of tissue which would normally have formed part of the epiphysis of the fibula. In histological sections such enlarged condyles appeared perfectly homogeneous with the rest of the femur and the distribution of the chondroblasts gave no indication that they were derived from more than one centre, fig. 11.

Plate 16. There was evidence that in some of those explants where the cut was made across the lower end of the opaque patch tibial tissue was similarly incorporated in the femur. As stated above, sections showed that the larger independent nodules were more sharply defined and more abruptly separated from the femur than the smaller nodules. It is probable, therefore, that unless the cut is made at a certain distance below the femur rudiment, so that the fragments of both tibia and fibula rudiments are above a certain minimum size, joint-formation cannot take place. The fact that the end of the fibula became incorporated with the femur more readily than the tibia may possibly be due to the smaller size of the fibula.

This apparent incorporation with the femur of chondrogenic tissue normally destined to form part of the tibia and fibula made it a little more difficult to determine the site of the future knee-joint with reference to the lower end of the opaque patch. From a careful study of the drawings made during the life of the cultures and of the histological sections it was, however, possible to define the position fairly accurately. An analysis of the results showed that the distal end of the opaque patch in buds up to 2 mm in length extends approximately to the end of the femur area and slightly between the proximal ends of the tibia and fibula areas. In buds of 3·1 mm the opaque patch extends a little farther between the areas of the tibia and fibula, but still does not pass much beyond the limits of the future epiphyses of these bones. Thus, the distal end of the opaque patch during this period of development does not greatly shift its position relative to the site of the future articular line.

On the other hand, the ten explants in which the cut was made proximal to the opaque patch (six from Group *c* and four from Group *d*) demonstrated clearly that the proximal end of the patch varied considerably in its relation to the site of the knee-joint according to the stage of development of the original bud. The most developed bud of this series was 3·05 mm in length and the skeletal rudiment was cut slightly proximal to the opaque patch. This explant developed into a femur from which only the condyles were missing. Another explant, from a 1·78 mm bud from Group *d*, had been cut in the same place relative to the opaque patch as the previous specimen, but formed only the proximal third of the femur; the distal part of the femur along with the tibia and fibula developed in the corresponding distal fragment of the same skeletal rudiment which was cultivated in the same watch-glass. In all the remaining explants of the series the femur was defective, the extent of the defect depending upon the size of the original bud and upon the

position of the cut with reference to the opaque patch. That is to say, the opaque patch covered a much larger proportion of the femoral area during the earlier stages of development than at a slightly later stage.

2. *Distal Fragments*—Four specimens were cut distal to the opaque patch (one from Group *b* and three from Group *c*) and consisted of a tibia and a fibula, with tarsal elements. Femoral fragments were absent. The fibula was clearly incomplete in one specimen, but in the other three it was not possible to decide whether the tibia and fibula were defective or not, although examination of the corresponding middle fragments from the same skeletal rudiments grown in the same watch-glasses suggested that the extreme proximal ends of the cartilages were probably missing.

Three explants (one from Group *b* and two from Group *c*) were cut across the distal end of the opaque patch. One of these, from a 1·74 mm bud, has already been referred to in the previous section; it developed into a tibia and fibula which were separated by a well-marked joint from a fragment of femur, consisting of one condyle and part of the other. The other two explants, from a 2·31 mm and 3·05 mm bud respectively, formed no femoral fragment and examination of the explants of the middle region of the same skeletal rudiments indicated that both the tibia and fibula of the distal explants lacked their proximal extremities.

Six explants (five from Group *b* and one from Group *c*) were also cut across the distal end of the opaque patch, but the cut was made slightly more proximal than in the preceding three cases. The buds from which the rudiments were taken ranged in length from 2·26–2·87 mm. No femoral fragment was formed by any of the explants. In one explant from a 2·87 mm bud the fibula was obviously incomplete and in some of the other specimens a study of the explants of adjacent portions from the same skeletal rudiments suggested that the extreme proximal ends of the tibia and fibula in the distal explants were missing.

Six explants (from Group *e*) were cut immediately proximal to the opaque patch. The explant from the smallest bud (1·35 mm) was stunted and distorted. It contained a tibia and fibula separated by a joint from a fragment of femur of about equal length; the distorted condition made it impossible to determine what proportion of the femur this fragment represented. Two specimens from 1·96 mm buds consisted of tibia, fibula, and about two-thirds of the femur, one from a 2·09 mm bud, in which the cut was made across the proximal end of the opaque patch, consisted of tibia, fibula, and the entire condylar end of the

femur, one from a 2·31 mm bud contained tibia, fibula, and about one-third of the femur, and one from a 2·35 mm bud, in which the cut had again been made just across the proximal apex of the opaque patch, formed tibia, fibula, and condylar end of the femur.

In five explants (four from Group *d* and one from Group *e*) the cut was slightly more proximal than in the preceding cases. Three of the rudiments were from 1·78 mm buds; one of these contained, in addition to the tibia and fibula, about two-thirds of the femur, whilst the other two had formed about three-quarters of the femur. In each case the missing part of the femur was formed by the explant of the proximal fragment of the same skeletal rudiment, as described in the previous section. The two remaining explants were from 2·39 and 2·48 mm buds, and each contained the condylar end of the femur in addition to the tibia and fibula.

In all those explants in which part of the femur was formed it was separated from the tibia and fibula by a well-marked joint. It was not possible to discover whether femoral tissue could become incorporated with the tibia and fibula in the same way that fibular and probably tibial tissue can be incorporated with the femur.

The results obtained from the cultivation of the proximal fragments of the skeletal rudiments, as shown by the foregoing description, confirm those obtained from the cultivation of the distal fragment. That is to say, during the 1·35–3·05 mm stages, the distal apex of the opaque patch does not alter very greatly in its relation to the site of the future knee-joint. On the other hand, the proximal apex varies greatly in this relation, being much more remote from the site of the knee-joint during the earlier stages than it is during the later stages; thus a fragment from a 1·78 mm bud cut slightly proximal to the opaque patch, fig. 31; fig. 12, Plate 16, forms about three-quarters of the femur in addition to the tibia and fibula, whilst one from a 2·48 mm bud, cut in the same place with reference to the opaque patch, forms only the condylar end of the femur, fig. 32; fig. 13, Plate 16. This alteration in the relation of the proximal end of the opaque patch to the site of the knee-joint is due partly to a proximal shrinkage in area of the opaque patch and partly to the progressive enlargement of the limb-rudiment.

3. *Middle Fragments*—Six explants (from Group *c*) were made of the middle region of the skeletal rudiment containing the opaque patch.

Four of these specimens, from buds ranging in length from 2·18–2·31 mm, formed the condyles and part of the shaft of the femur, whilst separated from the femur was a small, independent nodule of cartilage.

Two explants from rather more advanced limb-buds, 2·91 and 3·08 mm in length respectively, consisted of a single bilobed mass of cartilage with no independent nodules.

The drawings of the living cultures indicated that in at least two of the four specimens containing an independent nodule, fig. 33; fig. 14,

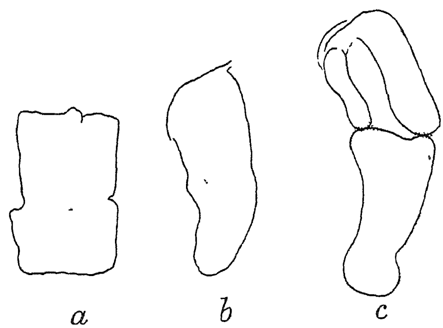


FIG. 31—Camera lucida drawings of the living distal fragment of a blastema from a 1·78 mm bud. (*a*) Immediately after explantation. (*b*) After 48 hours' cultivation. (*c*) After 4 days' cultivation; the tibia, fibula, and about three-fourths of the femur have been formed. A section of this explant is shown in fig. 12, Plate 16.

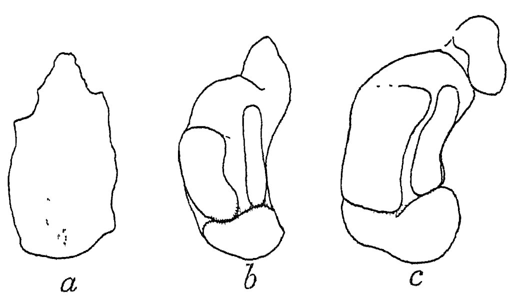


FIG. 32—Camera lucida drawings of the living distal fragment of a blastema from a 2·48 mm bud. (*a*) Immediately after explantation ; note that the cut has been made in the same position relative to the opaque patch as in the explant shown in fig. 31. (*b*) After 48 hours' cultivation. (*c*) After 4 days' cultivation; part of the metatarsus, the tibia and fibula but only the condylar end of the femur have been formed. A section of this explant is shown in fig. 13, Plate 16.

Plate 16, and in both those consisting of a single bilobed mass, tissue normally belonging to the tibio-fibula part of the skeletal rudiment had been incorporated with the femoral condyles.

Conclusions—(1) By using the opaque patch as an indication mark, it is possible to identify the knee-joint region at different stages in the

development of the early skeletal blastema. The results are summarized in the accompanying diagram, fig. 34.

(2) In fragmentation experiments small masses of tibio-fibula tissue become incorporated with the femoral condyles, so that a joint is only formed when a minimal amount of the tibio-fibula region is associated with the femoral region. It is not known whether the converse is also true.

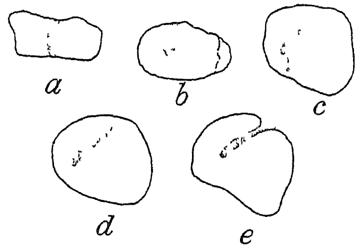


FIG. 33—Camera lucida drawings of the living middle fragment of a blastema from a 2·31 mm bud. (*a*) Immediately after explantation. (*b*) After 24 hours' cultivation. (*c*) After 48 hours' cultivation, showing an oblique extension of the opaque patch. (*d*) After 3 days' cultivation; the right arm of the opaque patch is beginning to disappear. (*e*) After 4 days' cultivation; the right arm of the opaque patch has disappeared completely and two nodules only have been formed, the larger representing the condylar end of the femur and probably part of the fibula, and the smaller probably representing the proximal end of the tibia. A section of this explant is shown in fig. 14, Plate 16.

6—THE BEHAVIOUR *in vitro* OF THE ISOLATED KNEE-JOINT REGION

Object of Experiments—To find whether the presumptive knee-joint region of the skeletal blastema can form a joint in the absence of the shaft rudiments of the tibia, fibula, and femur.

Material and Methods—Explants were made of the knee-joint region from forty-four limb-buds ranging in length from 1·26–4·35 mm.

To define the position of the presumptive knee-joint at different stage of development, a diagram similar to fig. 34 was made from an analysis of the data obtained from the fragmentation experiments described in Section 6. This diagram was laid on the culture bench for reference. According to the usual routine one leg-bud from each chick used was drawn before dissection, with the aid of a camera lucida, the drawing was then compared with the figures in the diagram to see with which of the developmental stages illustrated it corresponded, and the position of the knee-joint region in the experimental bud relative to the opaque patch was ascertained by reference to the corresponding figure in the

diagram. By this means the opaque patch could be used as an indication mark and the position of the knee-joint region identified in each explanted skeletal blastema after the skin and myogenic tissue had been removed in the usual way.

The joint region was excised from each explant as it lay on the surface of the clot, and was cultivated separately, though in the same watch-glass

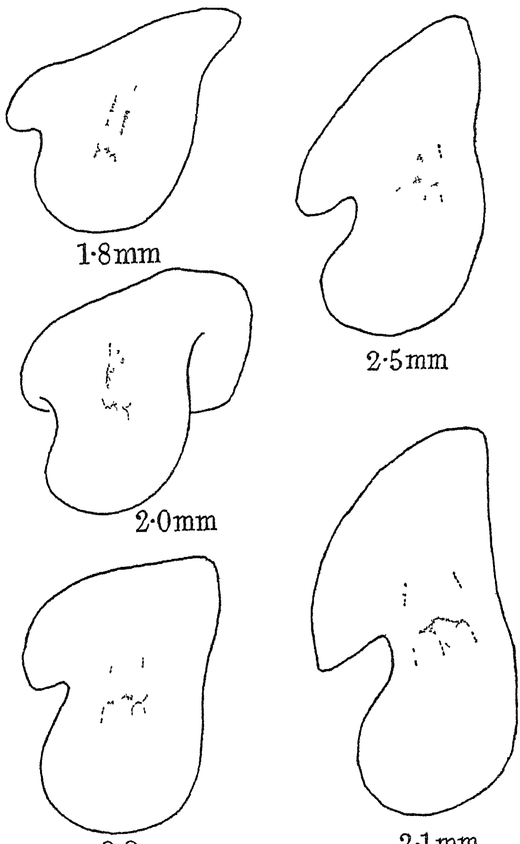


FIG. 34—Diagram illustrating the developmental potencies of the opaque patch at different stages of development.

as the rest of the blastema, for 4 days when the explants were fixed and sectioned. Camera lucida drawings of each fragment were made at the beginning of the culture period and thereafter at daily intervals. The proximal and distal fragments of the explants were used for the experiments recorded in Section 7.

Results—In the sections of twenty-two of the twenty-seven explants from 1·26–2·48 mm buds no sign of a joint could be found. Six of

these specimens consisted of a single bilobed nodule in which the division between the lobes was longitudinal with reference to the axis of the original limb-bud. In a study of the living cultures, however, it was noted that in two of the cases a transitory and imperfect attempt at joint-formation had been made during the earlier part of the culture period, all traces of which subsequently disappeared. Sixteen of the explants from the 1·26–2·48 mm buds consisted of two nodules of cartilage lying side by side and longitudinally divided in respect to the skeletal axis. No sign of a joint could be seen in any of the sections, but one explant had made an abortive attempt at transverse division during the first 48 hours *in vitro*. Of the remaining five explants from the 1·26–2·48 mm buds two consisted of a bilobed nodule in which a very faint indication of the articular line could be detected. Another was composed of two separate nodules, one of which showed a constriction. A study of this culture during life showed that a transverse division had begun to appear during the early stages of cultivation and had become quite marked. As development proceeded, one side of this transverse division disappeared and was finally represented only by the constriction mentioned above, but the other side persisted, and the two nodules seen in the section were thus transversely divided with reference to the axis of the limb-bud. A fourth

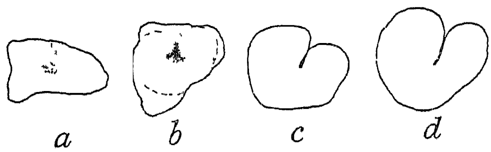


FIG. 35—Camera lucida drawings of the living joint region isolated from a 3·13 mm bud. (*a*) Immediately after explantation; the lower cut edge is the proximal one. (*b*) After 24 hours' cultivation. (*c*) After 48 hours' cultivation. (*d*) After 4 days' cultivation; a bilobed nodule has been formed, composed of femoral condyles and incorporated tibio-fibula tissue. A section of this explant is shown in fig. 15, Plate 16.

specimen consisted of two nodules longitudinally divided each of which showed a constriction at one end representing an abortive attempt at joint-formation. The fifth explant contained three nodules, the morphological significance of which was not clear.

Of the seventeen explants from buds ranging in length from 2·52–4·35 mm, eight formed bilobed masses of cartilage four of which had shown an abortive attempt at joint-formation during the early part of the culture period, fig. 35; fig. 15, Plate 16. Five consisted of two nodules separated longitudinally and all of them had made a transient and

incomplete attempt at joint-formation during the first two days *in vitro*. Two of the remaining cultures also contained two nodules lying side by side but traces of abortive joint-formation, fig. 36, could still be detected in sections of both specimens as a constriction of one or both nodules, fig. 16, Plate 16. Another explant consisted of the end of the femur partially fused with the end of the tibia, but separated from a fragment of the fibula by a joint. The last specimen, which was also the largest explant, was the only one which had formed a complete joint; it contained the condylar end of the femur clearly separated by an articulation from the proximal fragments of the tibia and fibula.

From the foregoing results it was clear first that the isolated knee-joint was unable to complete the process of joint-formation unless

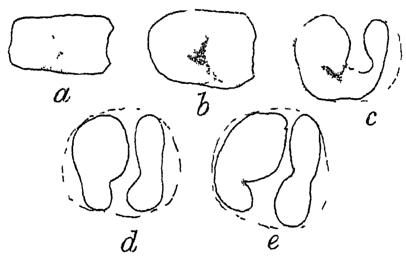


FIG. 36—Camera lucida drawings of the living joint region isolated from a 2·65 mm bud. (*a*) Immediately after explantation; the lower cut edge is the proximal one. (*b*) After 24 hours' cultivation. (*c*) After 48 hours' cultivation. (*d*) After 3 days' cultivation. (*e*) After 4 days' cultivation; two nodules have been formed which are longitudinally divided; each has a constriction near the proximal end which represents the boundary between a femoral condyle and the tibia and fibula respectively. A section of this explant is shown in fig. 16, Plate 16.

associated with a certain minimal amount of the long-bone rudiments and secondly that the explanted knee-joint region from older limb-buds showed abortive joint-formation during the early part of the culture period more frequently than explants from younger limb-buds.

It was interesting to note how presumptive tibio-fibula tissue became incorporated with presumptive femur tissue, as in some of the fragmentation experiments described in Section 5. In some cases, as recorded above, this process resulted in the formation of a single, bilobed mass of cartilage, fig. 35; fig. 15, Plate 16, whilst in others the tibial fragment became incorporated with one femoral condyle, and the fibula fragment with the other condyle, so that two separate nodules were formed lying side by side and divided longitudinally in relation to the skeletal axis.

Conclusions—(1) The isolated knee-joint rudiment has an extremely slight capacity for anatomical self-differentiation.

(2) This capacity increases a little with age.

(3) Complete joint-formation cannot take place unless a minimal amount of the tibia, fibula, and femur rudiments are included in the explant.

7—The Behaviour of the Presumptive Knee-joint Region as a Graft in the Tibio-fibula Region of another Skeletal Blastema

Object of Experiments—To find whether presumptive knee-joint tissue could form a joint if associated in an abnormal situation with presumptive shaft tissue.

Material and Methods—The skeletal rudiments from eleven pairs of limb-buds ranging in length from 1·91–3·74 mm were used for these experiments.

The position of the knee-joint region was identified by the method described in Section 6 and each pair of skeletal rudiments, divested of ectoderm and myogenic tissue in the usual way, were explanted in the same watch-glass on the surface of the clot. The knee-joint region was then excised from one rudiment and the tibio-fibular region of the other was cut in two about half-way along its length, so that the excised knee-joint tissue from the one rudiment could be pushed into position between the proximal and distal fragments of the other. The proximal and distal parts of the blastema from which the knee-joint tissue had been removed were used for the experiments described in Section 8.

The cultures were maintained for 4 days after which they were fixed and sectioned. Daily camera lucida drawings of the explants were made throughout the culture period.

Results—Four of the five grafts from 1·91–2·22 mm buds failed to become completely incorporated with the host tissue and the grafts, although fused with the proximal part of the host tibia and fibula, were separated from the distal fragments by connective tissue. Two of these grafts formed a bilobed mass of cartilage and two formed two nodules longitudinally divided, but none showed any signs of joint-formation. The fifth specimen, in which the graft had become completely incorporated with the host, was the only case in which the graft had been implanted immediately below the joint region of the host and not in the middle of the tibio-fibula region as in all other cultures. This graft had also com-

pletely failed to form a joint and had merged so perfectly with the host tibia and fibula that its original boundaries were impossible to distinguish.

The six explants from the older 2·87–3·74 mm buds were much better incorporated with the host cartilage than four of the five grafts of the younger tissue. All six were completely fused end to end with both the proximal and distal fragments of the host tibia, and in one explant with both fragments of the host fibula, though in the others fusion with the host fibula was either irregular or incomplete, fig. 37. It was interesting to find that in all these cultures the part of the graft which was fused with the host tibia had undergone complete, fig. 37, or, in one case, almost complete transverse division, whilst the fusion between host tibia and graft was so perfect that the original boundaries of the graft could usually only be ascertained by a careful study of the series of camera lucida drawings made during life. In section the appearance was merely that of a joint in the middle of the tibial shaft, fig. 17, Plate 16. The part of the graft fused with the host fibula had formed an articulation only in the single specimen, mentioned above, where there was an end-to-end fusion with both fragments of the host fibula. In two of the six implants, fig. 37; fig. 17, Plate 16, there was a longitudinal division between the part fused with the host tibia and that fused with the host fibula, in addition to the transverse division already described.

The results of these experiments showed that joint-formation in grafts of the presumptive knee-joint tissue only occurred where the graft was completely incorporated with the host cartilage. This confirmed the conclusion drawn from the experiments described in Section 6, viz., that a minimal amount of shaft tissue is essential for joint-formation in presumptive joint tissue, and it showed further that parts of the shaft remote from the normal joint are capable of exerting an effective influence on joint-formation. The single case in which the graft was fully fused with the host cartilage but formed no joint is perhaps explained by the fact that it was the only graft which had been implanted immediately below the host knee-joint region, so that there was shaft tissue on one side of the graft only.

Conclusions—(1) Association with presumptive shaft tissue is essential for joint-formation in presumptive knee-joint tissue.

(2) Presumptive shaft tissue remote from the normal joint region has the capacity for causing joint-formation in presumptive joint tissue.

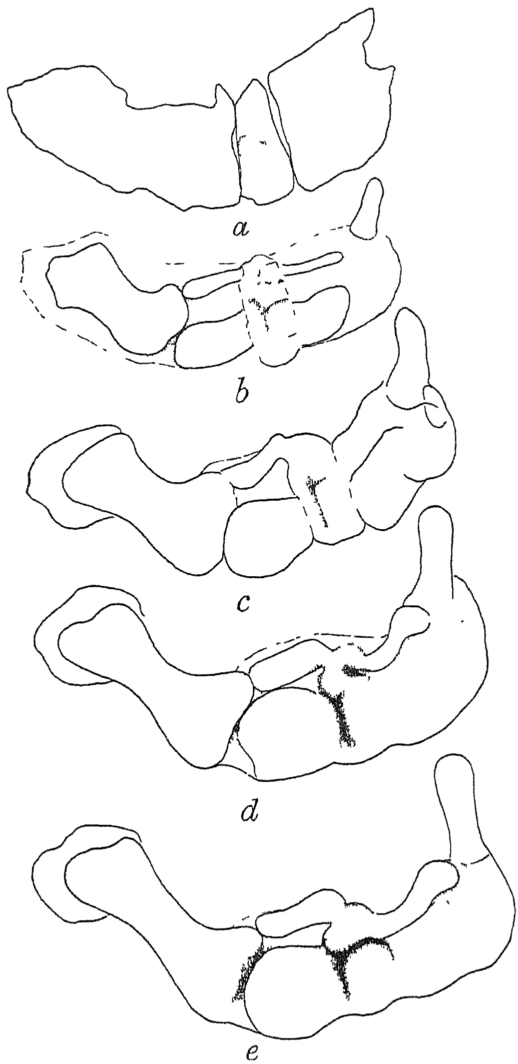


FIG. 37—Camera lucida drawings of a living blastema from a 3·61 mm bud; the isolated knee-joint region from the opposite leg-bud of the same embryo has been grafted into the middle of the tibio-fibula region and is orientated in the same direction as the host blastema. (*a*) Immediately after explantation. (*b*) After 24 hours' cultivation; the graft is already partly fused with the host tibia and fibula and shows indications of an articular line. (*c*) After 48 hours' cultivation; the relations of the graft with the host fibula have become distorted and the articular line has disappeared in the graft on that side. (*d*) After 3 days' cultivation. (*e*) After 4 days' cultivation; note the perfect incorporation of the graft with the host; the graft is longitudinally divided and has also formed a complete transverse joint across the part fused with the tibia but not across the part fused with the fibula. A section of this explant is shown in fig. 17, Plate 16.

8—The Effect upon Joint-formation of Excising the Presumptive Knee-joint Region

Object of Experiments—To find whether a joint could be formed in the absence of the presumptive joint tissue.

Material and Methods—The presumptive knee-joint region was excised from explants of seventy-four skeletal rudiments taken from limb-buds ranging in length from 1·26–4·35 mm.

The method of identifying the joint region and removing it from the explant has already been fully described in Section 6. As soon as the joint region had been cut out, the proximal and distal fragments were pushed into close apposition on the clot and were drawn with the aid of a camera lucida in the usual way. Drawings were also made at daily intervals and sometimes twice daily during the earlier part of the culture period.

Six of the explants were fixed and sectioned after two days' cultivation and the remainder after 4 days.

Six groups of experiments were made.

Group a—Twenty-three explants. Lengths of original buds: 1·44–2·65 mm. The knee-joint region was removed from the skeletal rudiments from both leg-buds of each embryo, and was cultivated separately (see Section 6).

Group b—Four explants. Lengths of original buds: 3·05–3·48 mm. A fairly large fragment was removed from the knee-joint region of one rudiment, whilst the opposite rudiment was explanted entire as a control. The excised joint tissue was cultivated separately (see Section 6).

Group c—Eleven explants. Lengths of original buds: 1·91–3·74 mm. The knee-joint region was taken from one rudiment and grafted into the tibio-fibula part of the opposite rudiment from the same chick (see Section 7).

Group d—Twelve explants. Length of original buds: 1·26–3·74 mm. The joint region was removed from one rudiment and cultivated separately (see Section 6) whilst the opposite limb-bud from the same chick was fixed and sectioned.

Group e—Twelve explants. Length of original buds: 1·74 (sub-normal), 2·91–3·96 mm. A narrow strip of tissue was cut from the joint region of one rudiment and rejected. A fairly broad fragment was cut from the joint region of the opposite rudiment from the same chick and cultivated separately (see Section 6).

Group f—Twelve explants. Length of original buds: 2·0–3·05 mm. The joint region was removed from one skeletal rudiment and rejected. The opposite skeletal rudiment from the same chick was cut across the middle of the tibio-fibula region, the distal part was turned upside down and the two fragments were then placed with their cut surfaces in such a way that the distal part of the tibial rudiment was in contact with the proximal part of the fibula rudiment and *vice versa*. In two of these twelve controls the distal fragment was wrongly orientated; in one case (rejected) the side of the distal fragment was placed in apposition instead of the cut surface, and in the other case the cut surfaces were in contact but the distal fragment was not turned upside down, so that the proximal and distal parts of the tibia were in contact with each other instead of with the fibular fragments.

Results—When the explants from which the joint region had been excised were examined 5–6 hours after operation complete fusion was seen to have taken place and the junction between the two parts was represented by a thin dark line. During the next 24 hours this dark line often became V-shaped, the apex of the V being directed distally, fig. 38, 1*b*.

After 48 hours *in vitro* it was usually very difficult to distinguish the site of the cut. At this stage of cultivation it was interesting to note that joint-formation began to appear in a large majority of the explants. The process was not quite so easy to follow as in the intact rudiments, as the greater part of the opaque patch which makes the normal joint so conspicuous had been removed by the operation. The process, however, was essentially the same, and sections of the six cultures (from Group *f*) fixed after 2 days *in vitro*, figs. 18 and 19, Plate 17, showed a histological picture that was almost identical with that of the developing knee-joint in the controls. In both the cells towards the ends of the long-bone rudiments were flattened and arranged with their long axes along arcs running parallel with the future articular surfaces. In places the site of the original cut could be faintly distinguished by the slightly wider intercellular spaces, but there was no necrosis. That the formation of a joint was not the result of trauma was shown by the fact that in the five controls in which the tibio-fibula region has been cut in half and the distal half turned upside down before being placed in apposition the apposed fragments of tibia and fibula had fused completely and showed no signs of the arcuate distribution of cells characteristic of joint-formation, fig. 19, Plate 17.

In Groups *c* and *f*, in which the blastema from one leg-bud was explanted with the knee-joint intact whilst the joint region was removed from the opposite blastema, a comparison of the two joints revealed an interesting fact. Although a joint had been formed in the operated rudiment in the absence of the presumptive joint-tissue, the end of the femur was

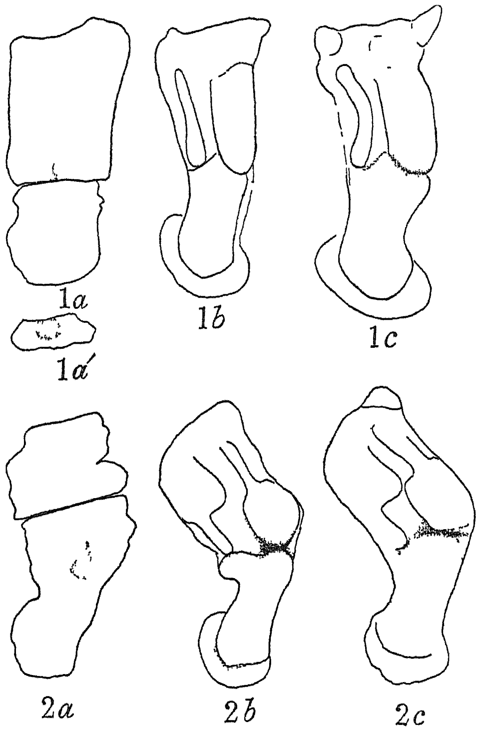


FIG. 38—1. Camera lucida drawings of a living blastema from a 2·7 mm bud after removal of the presumptive joint tissue. 1*a*. Immediately after explantation. 1*a*′. Excised fragment (rejected). 1*b*. After 24 hours' cultivation; a joint has begun to form, but the femur lacks condyles, *cf.* fig. 2*b*. 1*c*. After 48 hours, cultivation. 2. Camera lucida drawings of the living blastema from the opposite leg-bud of the same embryo ; the tibio-fibula region has been cut in half and the distal half reversed, so that the distal half of the tibial rudiment is in apposition with the proximal part of the fibula rudiment and *vice versa*. 2*a*. Immediately after explantation. 2*b*. After 24 hours' cultivation. 2*c*. After 48 hours' cultivation ; there is complete continuity between the tibia and fibula fragments. Sections of explants 1 and 2 are shown in figs. 18 and 19, Plate 17.

quite abnormal in shape, being considerably narrower than in the control, and lacking condyles, *cf.* fig. 38, 1*b* and 2*b*.

This effect could not be demonstrated in the tibia and fibula which have no conspicuous structure like the femoral condyles to enable a defect to be detected.

After 4 days' cultivation the separation of the articular surfaces was complete in those explants in which joint-formation had occurred. Sections of the sixty-eight cultures fixed after 4 days *in vitro* showed either a complete separation of both tibia and fibula from the femur, complete separation of the tibia but some degree of fusion of the fibula, or some degree of fusion of both tibia and fibula; it was very rare to find the tibia fused and the fibula independent.

In the six cultures of group *f* which were fixed after 4 days' growth four had formed a perfect joint between the femur and both tibia and fibula, figs. 20 and 21, Plate 17, whilst the other two showed very slight fusion of the fibula and femur but a complete joint between the tibia and femur. In five out of six controls fusion between both tibia and fibula fragments had occurred, figs. 22 and 23, Plate 17. Although fusion was usually so perfect in these controls that the site of injury could no longer be distinguished, the tibia and fibula fragments had each developed to their normal relative diameters, so that the tibio-fibula resulting from the fusion of two such fragments appeared as a thin rod of cartilage abruptly swelling to four or five times its original diameter half-way along its length. In the exceptional control referred to above, the cut had been made just below the knee-joint region instead of about half-way down the tibio-fibula part as in the other controls; in this specimen the proximal part of the tibia had fused with the distal part of the fibula, but the proximal part of the fibula had become incorporated with the femur to form an abnormally large condyle with which the distal part of the tibia articulated.

Whether or not a joint can be formed after the removal of the presumptive joint region was found to depend on two factors: (*a*) the amount of tissue removed, and (*b*) the degree of differentiation of the blastema at the time of operation.

The sixty-eight explants fixed after 4 days *in vitro* could be roughly classified in three groups according to the relative amount of tissue removed. Of the twenty-nine rudiments from which a comparatively small fragment had been excised, eighteen (62%) had formed a complete joint, ten (35%) had formed a perfect joint between the tibia and femur but showed some degree of fusion between the fibula and femur, and only one (3%) showed fusion of both tibia and fibula. In the twenty-four specimens from which a relatively larger fragment had been removed, five (21%) had formed a complete joint, eight (33%) had formed a complete joint only between the tibia and femur and eleven (46%) showed some degree of fusion between the femur and both tibia and fibula. None of the fifteen explants from which a comparatively large fragment

had been taken showed complete joint-formation, four (27%) had formed a joint between the femur and tibia only, and eleven (73%) showed fusion between the femur and both tibia and fibula.

The effect of removing a comparatively large fragment as compared with the effect of removing a much smaller fragment was rather strikingly illustrated by the experiments in Group *e*. In these experiments a narrow strip was cut from the articular region of one skeletal blastema and a broad fragment from the opposite blastema of the same embryo, the two explants being cultivated in the same watch-glass. The limb-buds used

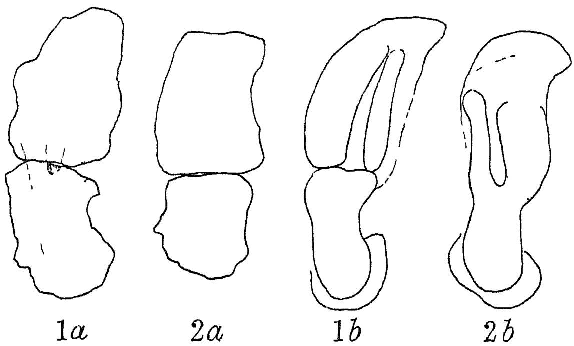


FIG. 39—Camera lucida drawings of a living blastema from a 3·05 mm bud after a thin strip has been excised from the articular region. 1*a*. Immediately after explantation. 1*b*. After 24 hours' cultivation; note that a joint has been formed. 2. Camera lucida drawings of the living blastema from the opposite leg-bud of the same embryo after a broad fragment had been excised from the articular region. 2*a*. Immediately after explantation. 2*b*. After 24 hours' cultivation; no joint has been formed and fusion between the femur and the tibia and fibula is already almost complete. Photographs of explants 1 and 2 before fixation are shown in figs. 24 and 26, Plate 18, and sections in figs. 25 and 27, Plate 18.

were, with one exception, at a comparatively advanced stage in which the position of the future joint could be distinguished even without reference to the opaque patch. In these explants a complete joint appeared in all those from which the narrow strip had been taken and was absent in all those from which a broad fragment had been removed, the fusion in the latter explants being usually so complete that all traces of the line of union had disappeared, fig. 39; figs. 24–27, Plate 18.

As previously stated above, the second factor which controlled joint-formation in the absence of presumptive joint-tissue was the degree of development of the blastema at the time of operation. It was noted that in completely unchondrified rudiments joint-formation would take place

after the removal of a relatively much larger fragment than would permit of joint-formation in a partly chondrified rudiment.

The results of these experiments demonstrated that the presumptive knee-joint tissue was not essential for joint-formation, and that mesoderm which would normally have developed into cartilage at some distance from the articular surface could be induced to form an articulation in the absence of the presumptive joint tissue. On the other hand, the presence of the presumptive joint tissue appeared to be essential for the development of the normal shape of the long-bone rudiments, as shown by the absence of the condyles after excision of the joint region. Thus although a joint was formed in the absence of the presumptive joint tissue, it lacked the specific characters of a knee-joint.

Conclusions—(1) Joint-formation can take place in the absence of the presumptive joint region.

(2) Joint-formation in the absence of the presumptive joint tissue is controlled by two factors: (*a*) the amount of tissue removed, and (*b*) the degree of development of the blastema at the time of operation.

(3) The articular ends of the long-bones cannot develop their normal shape after removal of the presumptive articular tissue.

9—Discussion

In considering the factors responsible for early joint-formation the results described in this communication enable us to eliminate several of the possibilities mentioned in the introduction.

The observations on the normal histogenesis of the knee-joint show that the articular tissue is chondrogenic like the rest of the blastema, so that joint-formation is not due to the presence of histogenetically different tissue at the future articular line. The experiments on the excision of the presumptive knee-joint region prove that, although the presumptive joint tissue is essential for the development of the normal shape of the knee, it is not esssential for the separation of articular surfaces; thus the potency for joint-formation is not rigidly localized in the limb mosaic. The cinema films of the development of the knee *in vitro* failed to demonstrate any signs of joint-formation taking place by mass movements of the cells in the knee region. Finally the mere fact that the knee-joint can develop in the explanted blastema shows that muscular, nervous, and vascular influences are not responsible for its appearance.

There remains the possibility that the separation of the articular surfaces is in some way the mechanical result of differential growth, and the

evidence obtained from the present study suggests that an explanation of this nature is correct.

As Carey (1922) has pointed out " centres of accelerated growth, segmentally arranged in an apparently continuous blastemal skeleton, are prior in time to the formation of joints." These " centres of accelerated growth " appear to correspond with the chondrogenic centres. Chondrogenesis begins and is always most advanced in the middle region of the long-bone rudiments and the shafts are chondrified at a stage when the joint region is still composed of undifferentiated mesoderm. Developing cartilage expands more rapidly than undifferentiated mesoderm because it increases in volume in three ways, viz., by the production of intercellular material, by cell division and by cell enlargement, whilst mesoderm inrceases by cell division only.

As Carey has pointed out it might therefore be expected that the soft tissues of the joint and perichondrium would present a resistance to the expansion of the chondrification centres, which would tend to flatten the undifferentiated cells and to orientate them with their long axes at right angles to the direction of pressure exerted by the expansion of the chondrification centres. This view is supported by the fact that in sections of both normal and explanted skeletal rudiments the cells in the less differentiated parts of the developing long-bones are somewhat flattened and are orientated in a direction roughly parallel with the future articular surfaces. Carey also noted this fact in normal development.

The expansion of the chondrification centres, the shape of which is determined by the limb-mosaic, would thus tend to compress the surrounding undifferentiated tissue into a kind of capsule around the cartilage. As chondrification and the differential growth which it produced spread into the epiphysial region of the two opposing elements such as the developing femur and tibia, more and more of the comparatively undifferentiated articular tissue would become flattened against, and incorporated in one or other of the adjacent long-bone rudiments. The final result of such a process would be the formation of an articulation.

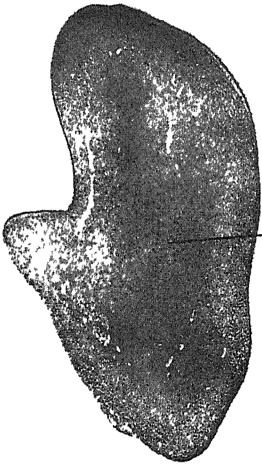
This hypothesis of joint-formation would explain all the experimental results obtained in the present study.

For example, it would be easy to understand why in fragmentation experiments where all or much of the femur is associated with very little, *i.e.*, with the least differentiated part of the tibio-fibula region, the presumptive tibio-fibula tissue should become incorporated with the femur instead of becoming separated from it by a joint. Murray (1926) states

that " it is quite certain that the limb-bud is a mosaic of regions determined in various directions (femur, foot, etc.), and it is probable that there do exist, between adjacent regions of the mosaic, boundary zones of tissues which can be converted into part of either of the two elements between which they lie, according to which regions gain control of them." The incorporation of tibio-fibula tissue with the femur is a direct confirmation of Murray's view and, according to the hypothesis of joint-formation expressed above, this incorporation would be due to the fact that the femur region of the blastemal fragment is able to " gain control " of all the undifferentiated tissue near the joint-region because it still possesses its most rapidly expanding part, whilst the tibio-fibula portion has been deprived of its most rapidly growing part by the operation.

That the association of undifferentiated tissue with rapidly growing chondrification centres is the essential factor in joint-formation is strongly indicated by the following facts, which the experiments described in this communication have demonstrated. (*a*) Explants of the presumptive joint-region cannot form a joint in the absence of a certain minimal amount of presumptive shaft. (*b*) The presumptive joint-region can form a joint when implanted as a heterotopic graft in another skeletal blastema, provided it becomes completely incorporated on both sides with presumptive shaft tissue, but not otherwise. (*c*) After the excision of the presumptive joint tissue a joint is formed, provided enough undifferentiated tissue is left behind. If all the undifferentiated tissue of the knee region is removed a joint does not form; this is shown by the complete fusion which takes place when a broad band of tissue is removed from the knee-region of an older, partly chondrified rudiment in which the region of undifferentiated mesoderm is much more restricted than in younger stages.

As shown in Section 4, only the earlier stages in joint-formation occur *in vitro* and the conditions of cultivation are not adequate either for the maintenance or for the further development of the articulation which eventually disappears by secondary fusion. Similar results were obtained by Pellegrini (1934) in chorio-allantoic grafts of partially differentiated avian joints. It seems probable that, although muscular movement is not concerned in the original appearance of the joint, it may be an important and possibly an essential factor in its subsequent development. It should therefore be emphasized that the results and conclusions contained in this communication refer only to the early stages of joint-formation, and that no consideration has been given in these experiments to conditions which may obtain in the later stages, when further changes, such as the formation of a joint cavity, take place.



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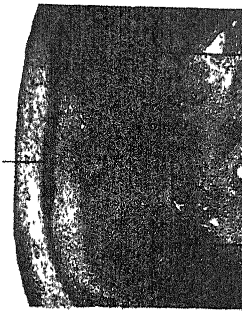

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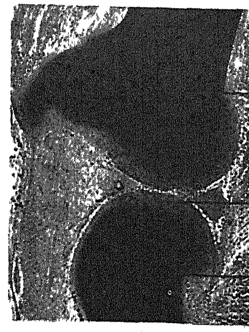

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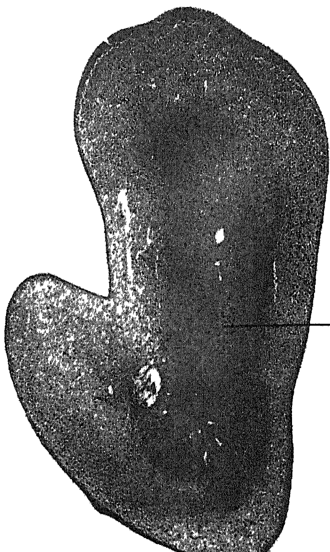

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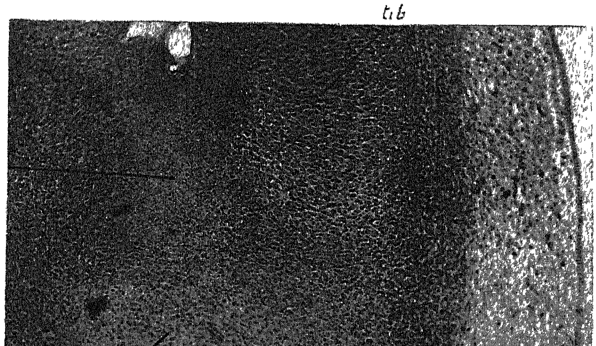

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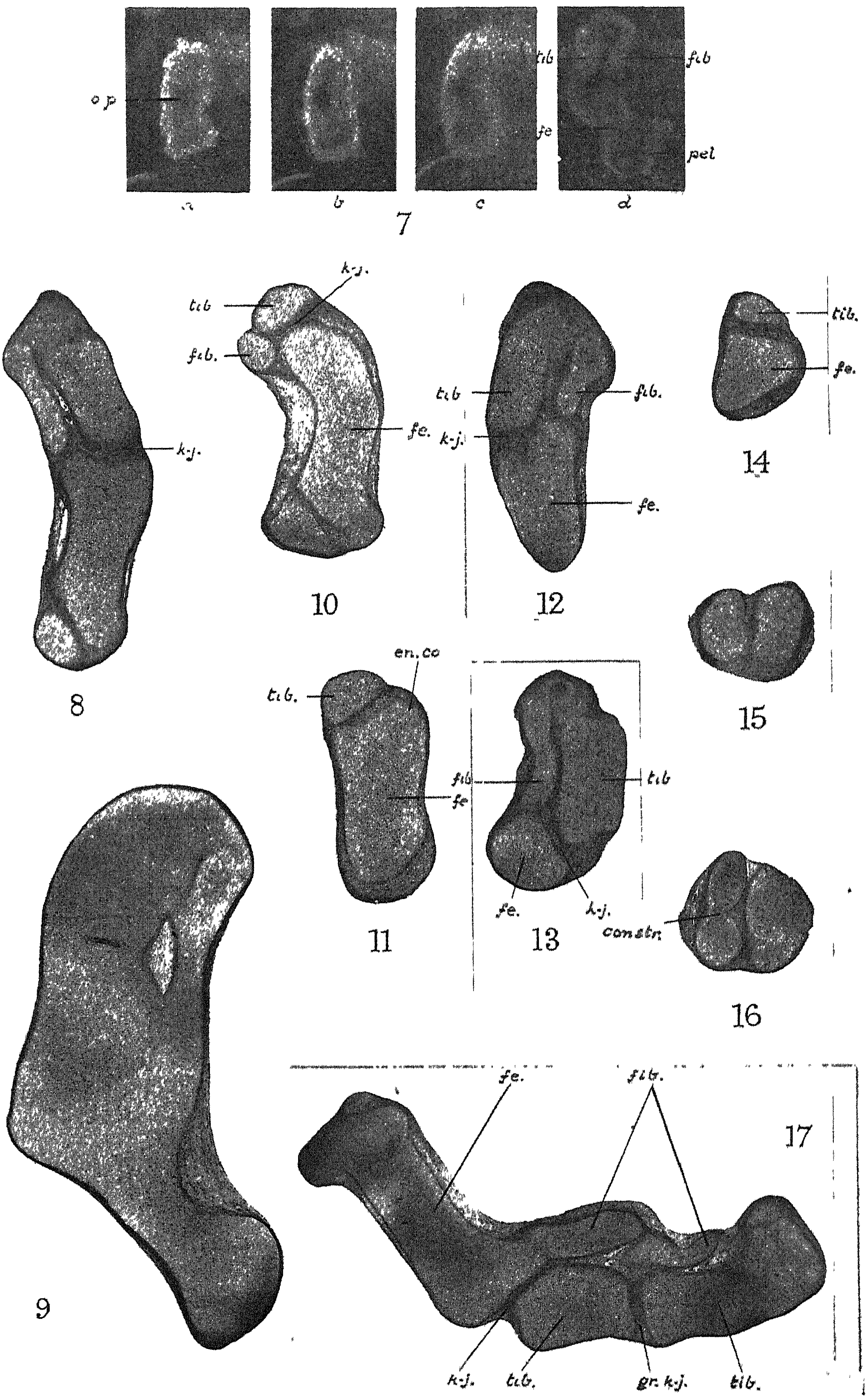
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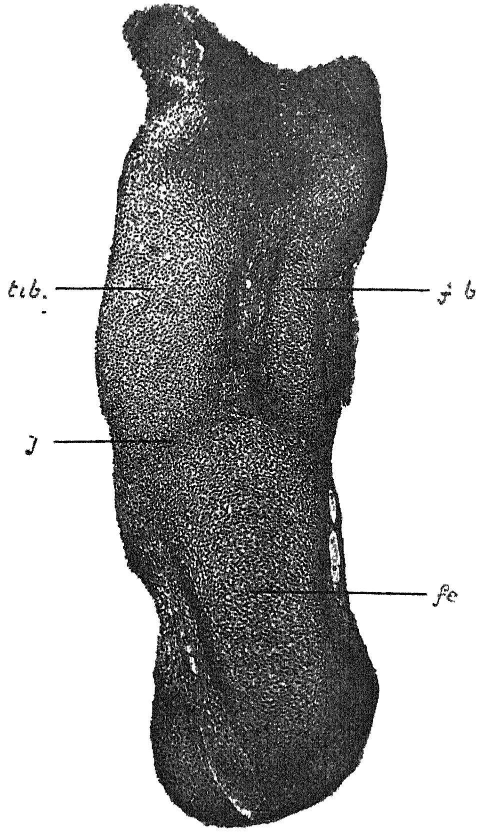

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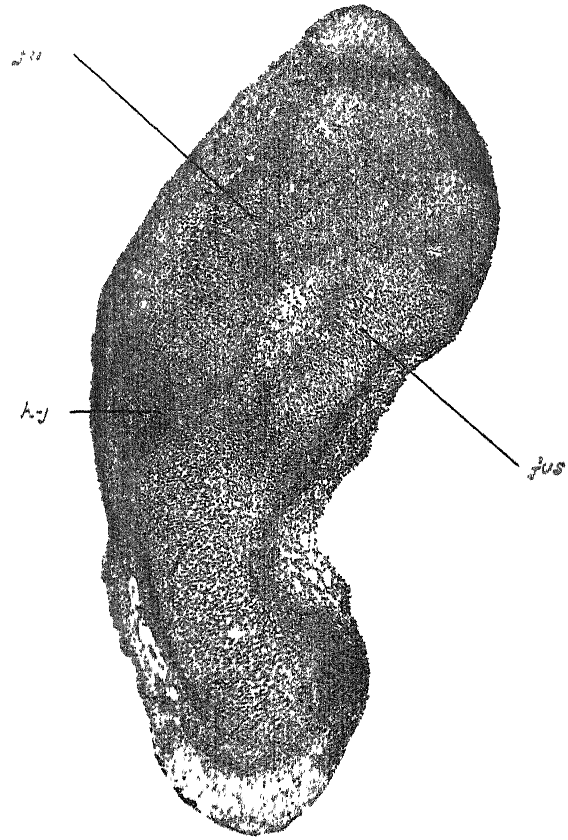

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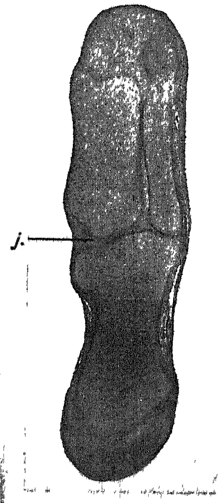

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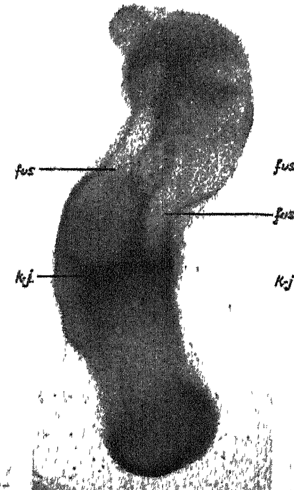

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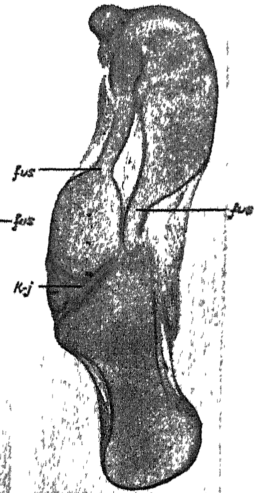

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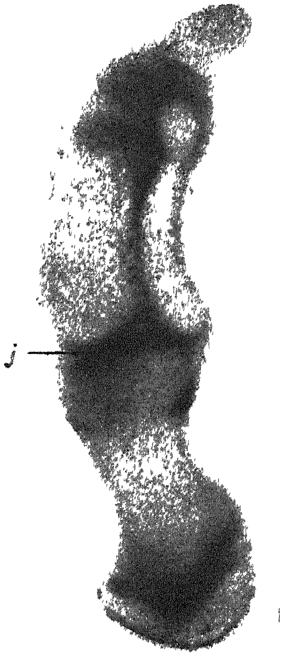

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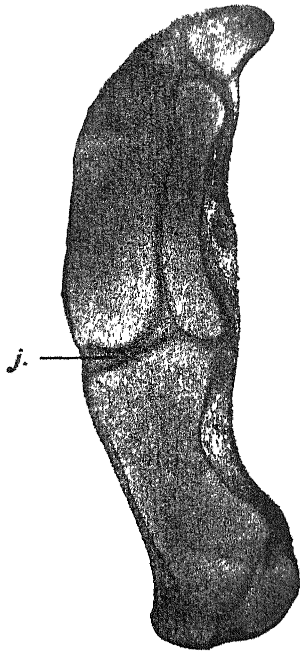

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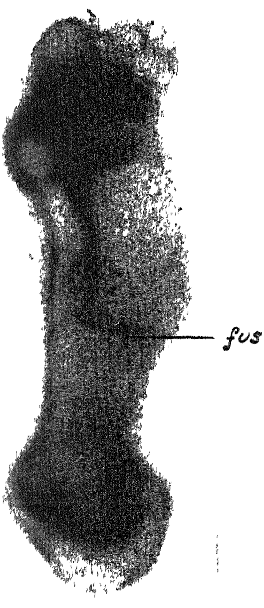

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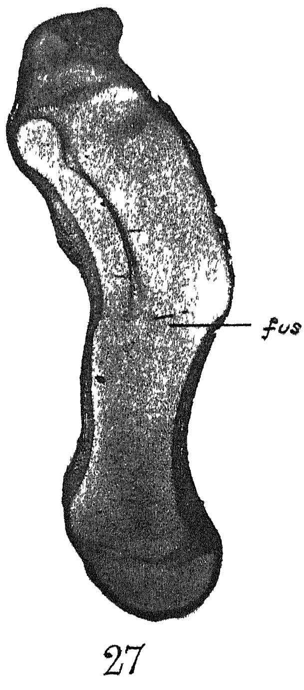

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The authors are deeply indebted to Dr. A. B. Appleton for some extremely valuable constructive criticism which was directly responsible for many of the experiments described in this communication.

They also wish to express their thanks to Mr. V. C. Norfield, who took most of the photomicrographs illustrating the paper, to Dr. P. D. F. Murray, Dr. F. G. Spear, and Dr. W. Jacobson for criticism of the manuscript and to the Medical Research Council by whom the expenses of the investigation were defrayed.

10—Summary of Results

The normal development of the knee-joint from the fourth to the tenth day of incubation is described.

The articular tissue in the normal limb-bud is chondrogenic like the rest of the skeletal blastema.

The skeletal blastema was removed from the leg-buds of 4-day fowl embryos and cultivated *in vitro* by the watch-glass method. The knee-joint developed in all such explants and its formation was studied by cinematography.

The cinema films afforded no evidence that the joint was formed by mass movements of cells in the knee-region.

The joint did not persist *in vitro*, but eventually disappeared by secondary fusion.

By means of fragmentation experiments *in vitro* the position of the knee-joint in blastemas at different stages of development was identified.

Explants of the isolated knee-joint region failed to form a joint.

The isolated knee-joint region when grafted into the tibio-fibula region of another explanted blastema formed a joint provided the graft became completely incorporated on both sides with the presumptive shaft tissue of the host.

Excision of the knee-joint region from an unchondrified blastema did not inhibit joint-formation, but the normal shape of the articular end of the femur was not produced.

Excision of the knee-joint region from a partially chondrified blastema did not inhibit joint-formation provided only a small part of the articular ends was cut away. If a large part of each of the articular ends was removed, complete fusion took place.

11—Conclusions

Joint-formation is not produced—

(*a*) as a result of the presence of non-chondrogenic tissue at the site of the joint;
(*b*) as a rigidly localized part of the limb mosaic;
(*c*) as a result of muscular, nervous, or vascular influence;
(*d*) by mass movements of cells at the joint region.

The separation of articular surfaces appears to be due to the mechanical result of differential growth, caused by the resistance of the undifferentiated tissue of the joint region and perichondrium to the expansion of the more rapidly growing chondrification centres.

The characteristic shape of the knee-joint is intrinsic in the limb mosaic.

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13—DESCRIPTION OF PLATES

Abbreviations

a.c., articular cartilage.
cap., capsule.
constr., constriction.
en. co., enlarged condyle.
fe., femur.
fib., fibula.
fus., fusion.
gr. k.-j., graft knee-joint.
j., joint.
k.-j., knee-joint.
o.p., opaque patch.
pel., pelvis.
tib., tibia.

Plate 15

Fig. 1—(× 32) Longitudinal section of a normal 2·3 mm limb-bud, showing the Y-shaped condensation of mesoderm representing the rudiment of the limb-skeleton. Note the elongated "opaque patch" containing many deeply stained degenerate cells. (Thionin.)

Fig. 2—(× 32) Longitudinal section of a normal 2·8 mm limb-bud. The skeletal rudiment is now much more distinct. (Thionin.)

Fig. 3—(× 84) Longitudinal section of the knee-joint in a normal 4·8 mm limb-bud showing the arcuate distribution of the cells at the articular end of the tibia. The tibia and femur are still continuous. (Thionin.)

FIG. 4—(× 32) Longitudinal section of the knee-joint of a $6\frac{1}{2}$ day embryo, showing the articulation of the femur and tibia. The epiphyses are almost fully chondrified and signs of transverse division have now appeared in the dense mesoderm of the joint region. The developing joint capsule is seen. (Thionin.)

FIG. 5—(× 32) Longitudinal section of the knee-joint of an 8-day embryo, showing the articulation of the femur and tibia. The articular surfaces are at this stage connected only by a little loose tissue. Note the densely cellular layer of articular cartilage in both femur and tibia. (Thionin.)

FIG. 6—(× 32) Longitudinal section of the knee-joint of a 10-day embryo, showing the articulation of the femur and tibia. The articular surfaces are now completely separate. (Thionin.)

PLATE 16

FIG. 7—Photographs from a cinema film of an explanted blastema from a 4-day embryonic limb. (*a*) Immediately after explantation ; note the opaque patch. (*b*) After about 12 hours' cultivation. The opaque patch has enlarged and has begun to assume a T-shape. (*c*) After 24 hours' cultivation. The opaque patch is now markedly T-shaped. (*d*) After 4 days' cultivation ; the knee-joint has become quite distinct and the pelvis, femur, tibia, and fibula are well developed.

FIG. 8—(× 24) Section of an entire explanted blastema from a 2·18 mm bud, see fig. 28, after 4 days' cultivation. Note the knee-joint which has developed *in vitro*. (Safranin; picro-indigo-carmine.)

FIG. 9—(× 24) Section of an entire explanted blastema from a 2·02 mm bud after 17 days' cultivation. The knee-joint which developed during the earlier stages of cultivation has completely disappeared as a result of secondary fusion. (Safranin; picro-indigo-carmine.)

FIG. 10—(× 24) Section of the proximal fragment of an explanted blastema from a 2·48 mm bud cut slightly distal to the opaque patch and fixed after 4 days *in vitro*, see fig. 29. The femur and the proximal ends of the tibia and fibula have been formed during cultivation; note the well-marked knee-joint. (Safranin; picro-indigo-carmine.)

FIG. 11—(× 24) Section of the proximal fragment of an explanted blastema from a 1·96 mm bud cut slightly distal to the opaque patch, see fig. 30, and fixed after 4 days *in vitro*. The femur and the proximal end of the tibia have developed but the proximal end of the fibula rudiment has become incorporated with the femur to form an enlarged condyle. (Safranin; picro-indigo-carmine.)

FIG. 12—(× 24) Section of the distal fragment of an explanted blastema from a 1·78 mm bud cut slightly proximal to the opaque patch, see fig. 31, and fixed after 4 days *in vitro*. About two-thirds of the femur and the tibia and fibula have been formed. (Safranin; picro-indigo-carmine.)

FIG. 13—(× 24) Section of the distal fragment of an explanted blastema from a 2·48 mm bud cut slightly proximal to the opaque patch, see fig. 32, and fixed after 4 days *in vitro*. The tibia and fibula have been formed, but only the condylar end of the femur. (Safranin; picro-indigo-carmine.)

FIG. 14—(× 24) Section of the middle fragment of an explanted blastema from a 2·31 mm bud cut immediately proximal and distal to the opaque patch, see fig. 33, and fixed after 4 days *in vitro*. Part of the femur and a smaller nodule

probably representing the proximal end of the tibia have developed during cultivation. (Safranin; picro-indigo-carmine.)

FIG. 15—(× 24) Section of the isolated knee-joint region of an explanted blastema from a 3·13 mm bud, see fig. 35, after 4 days *in vitro*. A bilobed nodule of cartilage had differentiated, but no joint has been formed. (Safranin; picro-indigo-carmine.)

FIG. 16—(× 24) Section of the isolated knee-joint region of an explanted blastema from a 2·65 mm bud, see fig. 36, after 4 days *in vitro*. Two nodules of cartilage have been formed which are separated by a longitudinal division. Each nodule shows a constriction representing the remains of an abortive attempt at joint-formation. (Safranin; picro-indigo-carmine.)

FIG. 17—(× 24) Section of an explanted blastema from a 3·61 mm bud into which the isolated knee-joint region from the opposite limb-bud of the same embryo, had been implanted half-way along the tibio-fibula region, see fig. 37. The specimen was fixed after 4 days' cultivation. Note the perfect incorporation of the graft with the host cartilage. A well-marked joint has developed in the part of the graft fused with the host tibia, but not in the part fused with the host fibula. These two parts of the graft are separated by longitudinal division. (Safranin; picro-indigo-carmine.)

PLATE 17

FIG. 18—(× 55) Section of an explanted blastema from a 2·7 mm bud from which the joint region had been excised, see fig. 38, fixed after 2 days' cultivation. Complete fusion has taken place, there is no necrosis at the site of the operation, and a joint has begun to form. Note the arcuate arrangement of the cells at the articular ends of the femur, tibia, and fibula as in the control explant, fig. 19.

FIG. 19—(× 55) Section of an explanted control blastema from the opposite bud of the same embryo, see fig. 38, fixed after 2 days' cultivation. The tibio-fibula region was cut in half and the distal half reversed so that the proximal cut surface of the tibia was in contact with the distal cut surface of the fibula and *vice versa*. Complete fusion between the tibia and fibula fragments has taken place. (Safranin; picro-indigo-carmine.)

FIG. 20—Photograph of a living explanted blastema from a 2·91 mm bud from which the joint region had been excised, after 4 days' cultivation. Note the well-developed joint which has appeared.

FIG. 21—(× 24) Section of the same culture after 4 days *in vitro*. (Safranin; picro-indigo-carmine.)

FIG. 22—Photograph of a living explanted control blastema from the opposite bud of the same embryo after 4 days' cultivation. The tibio-fibula region had been cut in half and the distal half reversed as in the explant shown in fig. 19. Note the perfect fusion between the tibia and fibula fragments.

FIG. 23—(× 24) Section of the same culture after 4 days *in vitro*. (Safranin ; picro-indigo-carmine.)

PLATE 18

FIG. 24—Photograph of a living explanted blastema from a 3·05 mm bud from which a narrow strip of tissue had been excised from the joint region, see fig. 39. A well-formed joint has developed (photographed after 4 days' cultivation).

FIG. 25—(× 30) Section of the same culture fixed after 4 days *in vitro*. (Safranin; picro-indigo-carmine.)

FIG. 26—Photograph of the living explanted blastema from the opposite limb-bud of the same embryo, see fig. 39; a relatively large fragment had been removed from the joint region, and the explant was then cultured for 4 days. No joint has been formed and the tibia and fibula have developed in continuity with the femur.

FIG. 27—(30) Section of the same explant fixed after 4 days' cultivation. (Safranin; picro-indigo-carmine.)

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The Function of Sympathetic Nerves in Relation to Skeletal Muscle—Evidence for Humoral Action

By O. W. TIEGS, from the Baker Research Institute and the Zoology Department, University of Melbourne

(*Communicated by W. E. Agar, F.R.S.—Received September* 17, 1934)

[PLATES 19 and 20]

§ 1—INTRODUCTION

In a previous paper (Corkill and Tiegs, 1933) evidence was given that the increase in strength of contraction which occurs in fatiguing skeletal muscle when its sympathetic nerves are stimulated (Orbeli's effect) exhibits certain features which point to a humoral mode of origin. This humoral action is suggested (i) by the persistence of the effect long after sympathetic stimulation has ceased, (ii) by an increase in latent period of onset of successive responses when they progressively weaken, (iii) by the fact that the effect may be reproduced by appropriate treatment with adrenaline.

A purely vascular origin of the effect having been excluded, the humoral interpretation would seem to offer a means of reconciling the occurrence of the phenomenon with the apparent absence of a direct sympathetic supply to the muscle tissue; for the only sympathetic nerves in muscle whose existence is beyond dispute are those on its blood vessels.

Inspection of records of well-defined Orbeli effects seems now to show that the chemical intermediary is indeed released at an appreciable distance from its site of action on the muscle; for the latent period of

onset is considerable (at least 10 seconds), while a much longer period may intervene between the cessation of stimulation and the attainment of maximal effect. These features are seen with various degrees of clearness in the tracings of the previous paper. They appear to a unique degree in an experiment of which fig. 1 is a record, and which is given here because it is the most powerful single response that has yet been obtained in this laboratory. The record is from the perfused sartorius, stimulated rhythmically through the ventral roots with maximal break-shocks, at a rate of 15 per minute. Stimulation of the sympathetic for rather less than 1 minute yields an effect of about 6 minutes' duration.

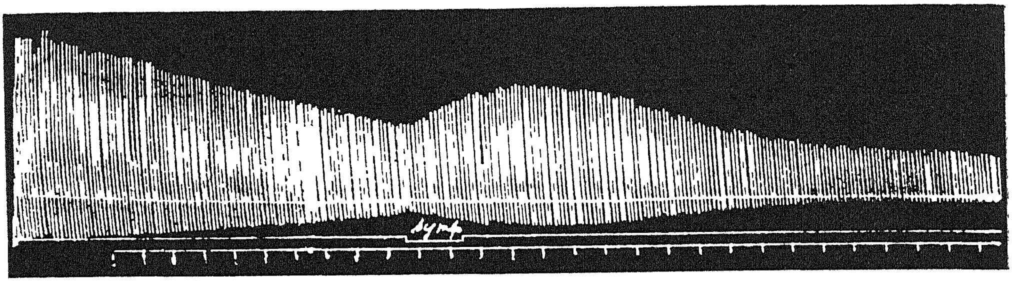

FIG. 1—Record from perfused sartorius, stimulated with maximal break shocks through eighth and ninth ventral roots; rate 15 per minute. Showing exceptionally powerful response to sympathetic stimulation. Time in half-minutes. Temperature 23° C.

The latent period of onset is about 12 seconds, while the response does not attain a maximum till as much as about a minute and a half after the cessation of stimulation. It is evident that such time relations are to be expected if the effect is caused by the sudden discharge of a chemical substance at localized places in the muscle (such as the walls of its arterioles), thence to diffuse on to the adjacent muscle fibres.

As in the previous work, this and all other experiments have been done on the frog (*Hyla aurea*). Details of method are given in the paper above referred to.

§ 2—THE OCCURRENCE OF ORBELI'S EFFECT AND ITS RELATION TO THE ADRENALINE SENSITIVENESS OF MUSCLES

The remarkable variability in adrenaline sensitiveness of muscles from different frogs was recorded in the previous paper, and reference was also made to the uncertainty with which Orbeli's effect is to be obtained. It is important for our hypothesis to determine whether the

capacity of a muscle to respond to sympathetic stimulation is in any way connected with its sensitiveness to adrenaline.

The procedure has been to record the Orbeli response and to determine the concentration of adrenaline which will produce in the same muscle an effect of about equal size. The minimum concentration of adrenaline to which the muscle will respond is also determined.

The method employed is as follows. The skinned hind-limb preparation, with sympathetic and ventral roots prepared, is set up with perfusion in the usual way. The sartorius tendon is freed and connected with the isotonic lever; but as pure sartorius contractions are not required, no precautions are taken to free the muscle from adjacent ones. This has an advantage over the gastrocnemius preparation; for while the latter usually fatigues fairly quickly with complete cessation of contraction, in the former a steady fatigue level is soon attained (*cf.* figs. 2, 7, 9, 10), at which the muscle may contract steadily for an hour or even much longer. On this level a fairly accurate comparison can usually be made between the effects of sympathetic stimulation and of successive perfusions into the muscle of adrenaline of known concentration. In addition possible errors due to faulty dilution of adrenaline can readily be checked by frequent repetition. The experiments have been made at room temperatures from 16–23° C.

An example of the type of record used for making the comparison is shown in fig. 2. This was from an exceedingly sensitive muscle capable of reacting to adrenaline at a concentration as low as 1 : 200 million. It is evident that here the response to sympathetic stimulation is much weaker than that yielded by adrenaline 1 : 40 million, but is rather stronger than that given by adrenaline 1 : 100 million.

A series of 23 experiments has been performed,

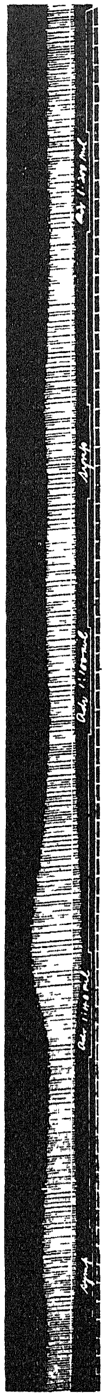

FIG. 2—Record from perfused sartorius, stimulated with maximal break shocks through eighth and ninth ventral roots; rate 15 per minute. Showing effects of sympathetic nerve stimulation and of perfusion of adrenaline at various concentrations through the muscle. The five effects are in response to (i) sympathetic stimulation, (ii) 1 : 40 million adrenaline, (iii) 1 : 100 million adrenaline, (iv) sympathetic stimulation, (v) 1 : 200 million adrenaline respectively. Time in half-minutes. Temperature 23° C.

which should be sufficient to permit of generalization. The results are given in Table I.

TABLE I

Experiment	Temperature °C	Response to sympathetic stimulation	Adrenaline equivalent of response million	Minimum effective concentration of adrenaline million
1	23	+ (very strong)	1 : 30 later 1 : 80	1 : 200
2	22	—	—	insensitive
3	22	—	—	1 : 1
4	21·5	+	1 : 15–20	1 : 20
5	23·5	+ (weak)	1 : 25	1 : 30–40
6	24	+ (very weak)	1 : 10	1 : 20
7	22·5	+	1 : 15	1 : 30
8	20	—	—	insensitive
9	20	+	1 : 15	1 : 20
10	21	+ (strong)	1 : 20–30	1 : 150
11	20	+ (strong)	1 : 5	1 : 40
12	20	+ (very weak)	1 : 15	1 : 15
13	21	+	1 : 20	1 : 30
14	21	+	1 : 20	?
15	19	+	1 : 5–10	1 : 10–20
16	18	+	1 : 10	1 : 30
17	19	—	—	1 : 1
18	18	+ (strong)	1 : 40–50	1 : 200
19	17	+	1 : 30	1 : 80
20	16	+ (weak)	1 : 30	1 : 30
21	18	+	1 : 10	1 : 20
22	18	+	1 : 15	1 : 20
23	16	—	—	insensitive

It will be seen that (i) those muscles that react only weakly or not at all to adrenaline do not respond to sympathetic stimulation; (ii) the muscles most sensitive to adrenaline give the strongest responses to sympathetic stimulation; (iii) the concentration of adrenaline required to give an effect equal to that obtained by sympathetic stimulation (adrenaline equivalent of sympathetic response), is, for an initial response, usually between 1 : 10 million to 1 : 30 million, though values well outside this range are sometimes met. For later sympathetic responses the adrenaline equivalent may be much lower (*cf.* experiment 1).

On the humoral hypothesis we should interpret these results as indicating that the strength of the Orbeli response is dependent on two factors:

(i) the concentration at which the chemical intermediary accumulates at its site of action (motor end-organs); (ii) the sensitiveness of the end-organs to the intermediary. For example, experiments 5 and 11 were made on muscles of about equal sensitiveness, each capable of responding to about 1 : 40 million adrenaline; the strong effect obtained in experiment 11 should be ascribed to the chemical intermediary reaching the motor end-organs at a much higher concentration, 1 : 5 million, than for the very weak effect of experiment 5, 1 : 25 million, only the most sensitive end-organs responding in this case.

This suggests that a failure of the Orbeli response to occur may be due either to impotence of the sympathetic or to insensitiveness—relative or absolute—of the end-organs. No example of the former condition has been encountered in this series of experiments, but an apparently quite definite case is given in § 5. With the insensitive muscles of Table I, the potency of the sympathetic was not tested, but in some later experiments, § 5, it has been examined for two muscles; in neither was there evidence of a potent sympathetic.

The adrenaline sensitiveness of the muscles used in this experiment requires comment. Corkill and Tiegs (1933) in a batch of 32 freshly captured frogs encountered only one instance of a muscle capable of responding to adrenaline at a concentration less than 1 : 10 million. In the present series, on the contrary, almost every muscle exceeded this in sensitiveness, two actually responding to 1 : 200 million. No explanation can be offered for this surprising variation. The method of testing was identical in both cases; many of the experiments were performed at the same time of year, early summer, as for the previous experiments, on frogs captured in the same locality.

§ 3 - The Formation of a Cardio-Augmentor Substance in Muscle by Means of Sympathetic Nerve Stimulation

Owing to its great sensitiveness to adrenaline, the isolated frog's heart has been extensively used in the present experiments to record the formation of an adrenaline-like substance in muscle when its sympathetics are stimulated. The experiments consist in steadily perfusing the heart with the perfusate—Ringer fluid—phosphate buffered—emerging from the veins of the hind limb (donor) preparation, and in observing the effect of prolonged sympathetic stimulation of the latter on the contractions of the former.

Method

(*a*) *Donor Preparation*—This is the skinned hind-limb preparation used in the foregoing experiments, except that the second sympathetic trunk is also prepared. The tip of the aorta cannula is inserted almost to the iliac bifurcation. All connections between sympathetic and aorta are severed. The renal portal vein having been ligated, the perfusate emerges entirely through the abdominal vein. A 5–6-inch head of Ringer fluid in the cannula usually suffices to maintain an adequate flow from the abdominal vein. The urinary bladder is excluded from perfusion by ligating the vesical veins, and sometimes even by excising it after ligating its cloacal end. The perfusate from the abdominal vein has therefore traversed the skinned hind-limb alone.

(*b*) *Heart Preparation*—The heart is removed from a freshly killed frog with only enough adjacent tissue to fasten it to the floor of a paraffin-lined glass dish, which is then completely filled with Ringer, fig. 3.

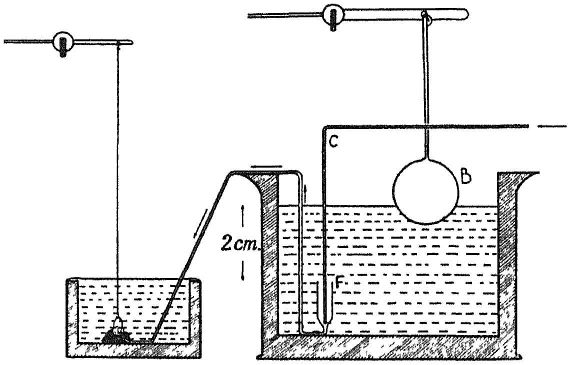

FIG. 3

Enough of the vena cava is left for the insertion later of a perfusion cannula. For perfusing the heart the following arrangement proved the most satisfactory, and was used for all the routine experiments. A glass dish holding about half a litre when filled to a height of about 6 cm serves as the reservoir from which the heart is perfused. The reservoir is so placed that the level of the fluid in it is about 15–20 mm above that in the dish containing the heart. Owing to the large bulk of fluid in the former, the requirements of the heart do not appreciably affect this head of pressure (venous pressure). For transferring the fluid to the heart a siphoning tube is made from a piece of $\frac{1}{4}$-inch glass tubing about 1 inch long, drawn out at one end into a rather wide capillary which is bent on

itself, so that, with the wide portion (funnel) F vertical on the floor of the reservoir, it can lead the fluid up over its rim down into the vena cava of the heart preparation, into which it is inserted and firmly tied.

The perfusate from the donor preparation is transferred to the heart by a capillary tube C about 5 inches long, bent near its middle at right angles, one end being tied into the abdominal vein of the donor frog, the other dipping into the tapering end of the funnel. If the perfusate emerges sufficiently fast, it completely displaces the fluid already in the lower end of the funnel, and reaches the heart almost undiluted. (This may readily be shown by adding a little methylene blue to the perfusing fluid.)

To examine the effect on the heart of known changes in composition of perfusing fluid, the fluid to be tested is allowed to siphon through a capillary into the funnel, the tube from the donor preparations being removed.

Contractions of the heart are recorded by a lever giving twenty-fold magnification. Unless otherwise stated, the experiments have been made at a room temperature of from 12 to 16° C.

The Heart Preparation as an Adrenaline Indicator

The freshly set up heart usually contracts powerfully; but the contractions soon weaken, gradually to recover again in about half an hour, after which the heart will generally run with wonderful regularity for hour upon hour. During the initial period it is apt to show some fluctuation. If this occurs at a later period, it is almost always due to insufficient "venous pressure," and can be corrected by raising the level in the reservoir. If it still persists, the heart must be discarded.

(*a*) *Action of Adrenaline*—If the heart be perfused with relatively strong adrenaline (1 : 1 million) in the above manner, there occurs a very powerful increase in strength of contraction, together, usually, with considerable acceleration. In the present work we are concerned with much milder effects. As the dilution becomes greater, acceleration is the first to disappear, and increases of 20% or more in amplitude of beat may occur without significant acceleration.

The sensitiveness of the heart to adrenaline varies enormously. Some hearts will not respond to adrenaline at a concentration lower than 1 : 100 million. Values of this order are given by Salant and Johnston (1924); but usually the sensitiveness is very much greater, and hearts that react to 1 : 1000 million are not uncommon. Sometimes a heart

is encountered capable of giving a just perceptible response to 1 : 4000 million adrenaline. It has been noticed that a freshly set up heart preparation is often, though not always, relatively insensitive to adrenaline, commonly failing to respond to a dilution of 1 : 100 million; but after an hour or two it gradually improves. A good adrenaline response often suffices to bring it out of its relatively insensitive condition; for example, a heart insensitive to 1 : 500 million adrenaline gave a strong response to 1 : 200 million, and was thereafter sensitive to 1 : 500 million, 1 : 1000 million, and 1 : 2000 million.

The above experiments were made at p_H 7·4. No certain change in sensitiveness could be detected over the range p_H 6·8–p_H 8, phosphate buffering. Parke Davis & Co.'s 1 : 1000 adrenaline solution, diluted immediately before application, has always been used; the results have been checked with the dry powder.

Fig. 4, Plate 19, which is a record from a heart in response to adrenaline at concentrations of 1 : 100 million, 1 : 500 million, 1 : 1000 million, and 1 : 2000 million respectively, will serve to show the degree of error which enters into estimations of adrenaline concentration made by this method. For example, the increase in amplitude of beat due to 1 : 500 million adrenaline is obviously greater than that due to 1 : 1000 million; but the difference is not sufficiently great for any accurate value to be assigned to a concentration of adrenaline that falls between them. The adrenaline concentrations recorded below are therefore only rough estimates. An exception must be made for estimations by the use of relatively insensitive hearts, 5B and 8A. In contrast to the very sensitive hearts these show an abrupt falling off in capacity to respond to adrenaline at higher dilutions. While in fig. 5B, Plate 20, for example, the response to 1 : 200 million adrenaline is strong, that to 1 : 300 million is only just perceptible; in fig. 8A, Plate 19, 1 : 300 million adrenaline is even without effect, although to 1 : 200 million the response is marked.

As an adrenaline indicator the heart preparation compares very favourably with other test organs which have been used for the purpose. The maximum sensitiveness of the frog's iris is stated to be 1 : 10 million (Ehrmann, 1905). Perceptible vaso-constriction in the perfused hind limb of the frog is stated to occur, in exceptional cases, even at a dilution of 1 : 10,000 million (Trendelenburg, 1915). A few experiments were made on this preparation, but the maximum sensitiveness so far encountered was 1 : 100 million; usually it has been much lower (1 : 20 million).

For the purpose of the present experiments the heart preparation displays one serious defect in that it often becomes markedly depressed, with loss of adrenaline sensitiveness, when the perfusate from the donor

is led into it. The depression is sometimes so marked that the heart must be discarded.

(*b*) *Hydrogen Ion Concentration of Perfusing Fluid*—This has been tested over a range p_H 8·2–6·2 ; appropriately buffered Ringer, as well as Ringer acidified with lactic acid were used. A change in hydrogen ion concentration from p_H 7·4 to 8·2 may be wholly without effect on the strength of beat or may produce mild depression. A change from p_H 7·4 to 6·5 or lower has always produced depression, often very severe, from which, however, the heart quickly recovers when Ringer at p_H 7·4 is perfused. Effects simulating weak adrenaline responses could therefore only occur with very large and easily detectable changes in hydrogen ion concentration of the perfusing fluid.

(*c*) " *Venous Pressure* "—The heart preparation is very sensitive to change in the rate of filling of the sinus, and a rise in level in the reservoir of only a few millimetres may yield an increase in strength of contraction. It was to overcome this serious source of error that the large reservoir was used. Since the vessel containing the heart is filled to overflowing the " venous pressure " is dependent only on the level of fluid in the reservoir. It seemed even desirable to record, along with the heart contractions, the height of fluid in the reservoir. A light glass bulb, B, fig. 3, floating on the surface, and giving an upward thrust to a lever magnifying about three-fold, was used for the purpose. In fig. 5B at x is shown the response of the heart to a 4 mm increase in height of fluid in the reservoir; increase in pressure head will, of course, read downwards.

Results

The general practice has been to test the adrenaline sensitiveness of the heart preparation about an hour after setting up; if it gives a good response to 1 : 200 million adrenaline it is retained for the experiment. The donor limb preparation is now made from a freshly killed frog, and the perfusate from the abdominal vein led into the funnel of the heart preparation. The usual effect is an increase in strength of contraction which falls off again in some minutes. Occasionally the opposite occurs, the heart being temporarily depressed. Not infrequently the coupling of the donor to the heart ultimately leads to a severe depression of the latter, when it must be discarded. But usually after some minutes the heart returns to its steady beat and may then continue so for hours. Fine platinum-tipped electrodes, connected with an induction coil, are

now fastened in position under the two sympathetic trunks of the donor. The fluid in the aorta cannula is maintained at a constant level. After the heart has been running steadily for at least 15 minutes, the sympathetics are given prolonged stimulation. Spread of current on to the spinal nerves is avoided.

A record from an experiment is given in fig. 5, Plate 20. About $2\frac{1}{2}$ minutes after commencement of stimulation, the heart contractions gradually gain in amplitude, attain a maximum after about $5\frac{1}{2}$ minutes, and then gradually decline again. Ten minutes later sympathetic stimulation produces a similar effect, and this occurs a third and fourth time. The lower record is a continuation of the same experiment. The cannula from the donor has been removed, and the adrenaline equivalent of the effect determined by perfusing adrenaline of known concentration in the manner above described. It is evident that the effect produced by sympathetic stimulation has, in this experiment, an adrenaline equivalent of rather less than 1 : 200 million.

The " venous pressure " is recorded above the heart tracing; change in this pressure is evidently not the cause of the effect. The efficiency of the pressure record is shown at x, where an increased strength of beat, much smaller than that due to sympathetic stimulation, has been produced by deliberately raising the " venus pressure " by an amount, 4 mm, which the lever can easily record.

In this experiment a very slight but apparently significant acceleration of 1–3 beats per minute (recorded below the time marker) accompanies the increase in amplitude, but as an adrenaline indicator this is evidently less efficient than the latter, and has therefore not been extensively used.

The experiment has for various purposes been performed 51 times: in only 10 did an effect fail to occur. Some failures are probably due to the use of insufficiently sensitive hearts. The strength of the effect varies in different preparations. That shown in fig. 5 is exceptionally strong; usually the increase in amplitude is smaller, and the ascent less steep, *cf.* fig. 6. The adrenaline equivalent has not in all experiments been determined. It undoubtedly varies greatly, and values as low as 1 : 1000 million or less have been recorded. These variations are to be expected, for apart from variations in the potency of the donors, there is much variation in the strength and frequency of the heart beat, necessitating a variation in the supply of perfusing fluid to it, with a consequent variation in the dilution of the chemical factor carried over from the donor preparation. In practice, hearts from rather small frogs have been used; if these were not available the amplitude has

been decreased by appropriate weighting of the lever. To increase the efficiency of the experiment, the height of fluid in the aorta cannula has been adjusted to deliver the perfusate at a rate only little in excess of the heart's requirements (determined by watching the rate of fall in the funnel when the main bulk of fluid in the reservoir has been removed).

Unless the effect is very weak it can be obtained many times in one and the same preparation. In one exceptional experiment of 8 hours' duration it occurred 23 times in succession ; the initial effects showed no obvious sign of fatigue, *cf.* fig. 5; but for later stimuli they weakened considerably and much longer stimulation of the sympathetic—as much as 7 minutes—was needed.*

Discussion

Change of " venous pressure " has already been excluded as a source of error. The following factors need consideration.

(*a*) It is conceivable that, with the experiment in its above form, sympathetic stimulation, by causing vaso-constriction, may retard the perfusion of the donor preparation. If the perfusate contains a cardio-inhibitory substance, then the heart, by using the fluid of the reservoir that has not traversed the donor preparation, may improve in strength of contraction. The objection is excluded on the following grounds: (i) the effect is still obtainable even when the reservoir is not present, the head of Ringer in the funnel being maintained at a constant height by pipetting off any excess over a marked level; (ii) sympathetic stimulation is not always attended by vaso-constriction in the muscle. A systematic study of the vaso-motor effects has not been undertaken; in the few experiments where it was sought vaso-constriction did not occur. The point has been tested in two ways—firstly, the rate of fall of perfusing fluid in the aorta cannula has been observed and found unaltered by sympathetic stimulation, in experiments where good effects on the heart were obtained; secondly, the rate of emergence of perfusate from the donor preparation has been determined with and without sympathetic stimulation in preparations which have shortly before and shortly afterwards given good effects on the heart. No change in rate of emergence occurred. Variable rate of flow of perfusing fluid is therefore not the cause of the effect.

* The maximum number of Orbeli responses obtained by Corkill and Tiegs (1933) in a single preparation was eight. I have since encountered a muscle in which, in a $3\frac{1}{2}$-hour experiment, 25 successive Orbeli effects occurred each in response to 1-minute stimulation of the sympathetic. Indeed, only in the last few effects was there any sign of marked fatigue of the sympathetic.

(*b*) If the effect is due to altered hydrogen ion concentration of the perfusate, the latter must change from a markedly acid reaction towards neutral. Such a change does not occur. About 10 drops of the perfusate in a preparation which shortly before and shortly after gave a good heart effect were collected with and without sympathetic stimulation, as they emerged from the abdominal vein. The hydrogen ion concentration of the two lots was determined with the capillary quinhydrone electrode.*

The following readings are illustrative :—

	p_H
Perfusing fluid	7·47
Perfusate before sympathetic stimulation	6·79
Perfusate after sympathetic stimulation	6·76

The effects are not, therefore, due to altered hydrogen ion concentration.

(*c*) *Accessory Chromaffine Tissue*—The formation of adrenaline from accessory chromaffine tissue is a much more serious source of error. This has necessitated a detailed examination of its distribution in this species of frog. For the purpose the particular regions to be examined are dissected under saline, and then immersed for a day in a solution consisting of 1 part potassium bichromate 5% solution, 10 parts formalin 5% solution (Wiesel). When such material is cleared in xylol the stained chromaffine cells appear bright golden on an almost colourless background. The tissue may be examined either in sections cut from paraffin, or in whole preparations mounted in Canada balsam. Since the stained cells are conspicuous, even in thick slabs of tissue, the latter method has been mainly used. The chromaffine tissue, apart from that on the kidneys, is found to have the following distribution. (i) *In the sympathetic system*—In the main sympathetic chain chromaffine cells are scarce. Occasionally single cells or a group or even several groups of two or three such cells are encountered in the ganglia, whilst isolated cells may even be present in the interganglionic nerve strands. In constrast to this, the ganglia of the cœliac (solar) plexus, together with the nerves that pass from these ganglia along the intestinal arteries, are very rich in chromaffine cells. In the urinogenital plexus, though prevalent, they are less numerous. In the ischio-coccygeal plexus (Gaupp) they do not occur. (ii) *In the spinal nerves*—Very rarely a chromaffine cell may be seen in a spinal nerve near the sympathetic chain. (iii) *On the walls of the veins*—On the renal veins chromaffine cells may sometimes occur either in clusters or singly. On the adjacent parts of the vena cava an

* These measurements were kindly made for me by Mr. A. Doutch.

isolated cell may occasionally be seen. Elsewhere they are absent. (iv) *On the walls of the arteries*—On the walls of the aorta itself they occur only rarely, but on its visceral branches they may be very numerous. They are present in considerable numbers on the walls of the cœliaco-mesenteric artery adjacent to the cœliac plexus, and extend well beyond the division of the artery into its cœliac and mesenteric branches. But on the smaller intestinal branches they are scarce. In the renal arteries they are abundant. Below this level they do not occur.

Such being the distribution of the accessory chromaffine tissue, it evidently cannot be a source of error in the present experiments, for (1) most of it (that on the cœliac arteries) is removed from the preparation; (2) the aorta cannula is inserted along that artery well below the lowest limits of the chromaffine tissue; (3) all connections between the aorta and the sympathetic have been cut; (4) the lower sympathetic ganglia, where adrenaline may arise from the few scattered chromaffine cells, are not perfused.

It seemed worth while to examine even the remote possibility that accessory chromaffine tissue might be lodged in the tissues of the hind limbs themselves. Accordingly the iliac arteries and their continuations have been examined along the length of the leg, but with negative results. The veins, nerves, and adjacent connective tissue are also free from chromaffine cells. In the urinary bladder they are also absent; and in any case the vesical veins have been ligated.

In view of the observation of Abel and Macht (1912) that the secretion from the poison (skin) glands of the toad (*Bufo agua*) is very rich in adrenaline, it should be emphasized that the precaution has been taken to remove every fragment of skin from the hind-limb preparation, for it is conceivable that a secretion might be ejected from surviving skin glands during sympathetic stimulation, and contaminate the perfusate.

§ 4—The Cardio-augmentor Substance as a possible Intermediary between Sympathetic Nerves and Muscle

It remains to determine whether the cardio-augmentor substance can be an intermediary between the sympathetic nerves and the muscle tissue, or whether it is a product of the effector cell itself. This has been examined in two types of experiment.

(i) It was noted (Corkill and Tiegs, 1933) that at temperatures below about 12° C sympathetic stimulation failed to give Orbeli's effect in fatiguing muscle, the responses to adrenaline being similarly paralysed, or, at any rate, weakened by cold. This provides a ready means of

separating the effect on the muscle from that transferred on to the heart; for the heart responses to dilute adrenaline are not obviously weakened by lowered temperature.

The preparation is set up as before, except that the ventral roots of the donor are also prepared. The behaviour of the muscle is examined by attaching the freed gastrocnemius tendon to the muscle lever. Heart and muscle are recorded simultaneously.

A record from an experiment is given in fig. 6. Commencement of rhythmical maximal contraction of the hind-limb musculature does not perceptibly affect the heart. When fatigue of the muscle has set in, the sympathetic is given prolonged stimulation. There is no effect on the muscle, but the heart beats gradually increase in amplitude. The cardio-augmentor substance cannot therefore be a product of the activity of the muscle tissue so far as this is manifested by an improvement in strength of contraction. (Contraction of plain muscle of blood vessels is also excluded as a source of the substance since there was no indication of vaso-constriction as estimated by rate of fall of fluid in the aorta cannula.)

Incidentally, the experiment answers a possible objection that may be brought against the earlier experiments of Corkill and Tiegs, namely, that the failure of Orbeli's effect to occur at low temperature might be due to the inactivity of sympathetic nerves in the cold, and not, as was supposed, to insensitiveness to adrenaline; for in the present experiments activity of the sympathetics is unimpaired.

(ii) Ergotoxine* 1 : 50,000 inhibits the normal responses of the muscle to sympathetic stimulation and to adrenaline. The adrenaline responses of the heart are much less affected. Sometimes, particularly with massive doses of ergotoxine (1 : 5000) the heart becomes powerfully depressed, and its adrenaline sensitiveness is then much weakened, though not abolished (it will, for example, still respond to adrenaline 1 : 1 million), but other hearts are not even noticeably depressed and their adrenaline responses may also be quite unaffected. For example, 10 minutes' exposure to ergotoxine 1 : 5000 failed appreciably to weaken the responses of a heart to adrenaline 1 : 200 million. This differential action of ergotoxine on the two tissues enables us to dissociate the effect of the sympathetic on muscle, from the transferred heart effect.

* The Burroughs Wellcome preparation was used. Its potency was tested on the guinea pig uterus, the response of which to adrenaline 1 : 1 million was inhibited in 10 minutes by 1 : 500,000 ergotoxine. I am indebted for these observations to Mr. H. Ennor. In the absence of a guaranteed standard for comparison, an actual assay could not be made.

A record from such an experiment, performed at 21° C, is given in fig. 7, Plate 20. The ventral roots of the donor are given rhythmical maximal stimulation, and the behaviour of the muscle recorded by attaching the liberated sartorius tendon to the muscle lever. Com-

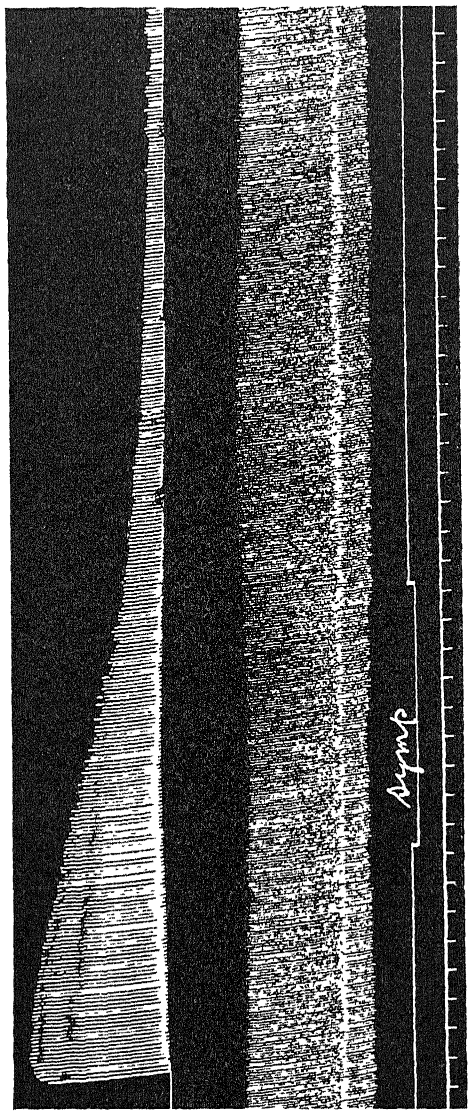

FIG. 6—Showing a response of the receptor heart in the absence of an Orbeli effect in the donor preparation. Upper record—gastrocnemius of donor; maximal ventral root stimulation; 15 per minute. Lower record—receptor heart. Time in half-minutes. Temperature 12° C.

mencement of contraction of the muscle of the donor does not perceptibly affect the heart. As fatigue develops, the sympathetics of the donor are given prolonged stimulation; there occurs an Orbeli effect in the muscle and after a latent period of 2 minutes the transferred heart effect commences. We require to know whether the substance which affects the heart is due to the muscular response, or whether it can be the substance which has produced this response. Accordingly at A, fig. 7, the Ringer fluid in the aorta cannula is replaced by Ringer containing 1 : 50,000 ergotoxine. After 6 minutes, sympathetic stimulation is repeated. The effect on the muscle does not occur; the heart responds as before. Effective poisoning of the muscle is further shown at B, fig. 7, where the contents of the aorta cannula are replaced by a 1 : 50,000 ergotoxine solution containing 1 : 1 million adrenaline. There is no effect on the muscle; the heart responds powerfully. (Contraction of the plain muscle of the blood vessels has not been specifically referred to as a source of the cardio-augmentor substance; it is excluded by the fact that this tissue has also been poisoned, the 1 : 1 million adrenaline solution running through the preparation without any indication of vasoconstriction.)

This experiment recalls that of Cannon and Bacq (1931) who obtained the transferred heart effect even after the pilomotor response had been inhibited by ergotoxine (cat); but the improvement in the effect after ergotoxine that occurs in the cat has not appeared in the present experiments.

§ 5—Potency of the Donor Preparation in Relation to the Adrenaline Equivalent of the Orbeli Response

Since the adrenaline equivalent of the Orbeli response varies considerably for different muscles, it was expected that the variability of potency of the donor preparation would be associated with this. Only a few experiments were undertaken to test this point, for it was soon evident that such a relation did not exist. The experiment consists in comparing for a series of preparations the adrenaline equivalent of a sympathetic response with the equivalent of the effect transferred on to the heart. As a single experiment is apt to be lengthy, it has been necessary to use a new receptor heart for each experiment. The error arising out of variable rate of perfusion of the donor, to supply the needs of different hearts, has been overcome by arranging a constant rate of perfusion of each donor preparation to deliver a perfusate in excess of the requirements of any heart. This is the reason for the low adrenaline

values recorded by the hearts. The values given in Table II were obtained:—

TABLE II

Experiment	Temperature °C	Adrenaline equivalent of Orbeli effect million	Sensitiveness of muscle million	Adrenaline equivalent of heart response million	Adrenaline sensitiveness of heart million
1	18	negative	1 : 20	negative	1 : 1000
2	20	1 : 30	1 : 50	1 : 1000	1 : 4000
3	18	1 : 40	?	1 : 1500	1 : 2000
4	19·5	1 : 5	1 : 40	1 : 2000	1 : 2000

The adrenaline equivalents of the heart effects are only very rough estimates; nevertheless, the fourth experiment, in which an Orbeli effect of high adrenaline value is accompanied by a just perceptible effect on the heart, seems to render further experiments on these lines superfluous.

Various explanations may be offered for this unexpected result: (i) the high adrenaline value of an Orbeli effect may not imply an exceptionally potent sympathetic, but may be dependent on the rate at which the intermediary can penetrate into the motor end organs, where it acts, before it becomes dispersed through the tissue; (ii) the magnitude of the transferred heart effect may again depend, not only on the potency of the sympathetic but also on the rate at which the intermediary can penetrate through the capillaries into the perfusing fluid. The contrast between the comparatively steep ascent in fig. 5 and the very gradual ascent in fig. 6 suggests that variable rate of diffusion does in fact occur. Destruction of the intermediary in the muscle may also be a factor concerned.

Experiment 1 is of interest: it shows a muscle of medium adrenaline sensitiveness which failed to respond to sympathetic stimulation. Even when the perfusion rate was reduced, there was no effect on the heart. It would seem to be an example of a normal muscle with an impotent sympathetic, as discussed in § 2.

The above method has also been used to test the potency of the sympathetic in muscles relatively insensitive to adrenaline. Two frogs only were used from a batch which proved to be relatively insensitive to adrenaline (probably owing to a fortnight's captivity in cold weather). One gave a just perceptible response to 1 : 7 million adrenaline, the other was insensitive. In neither did an Orbeli response occur, nor was there any effect on the heart (temperature 16° C).

§ 6—Transference of Orbeli's Effect from a Donor to a Receptor Muscle

Final conclusive evidence for the humoral origin of Orbeli's effect would be provided by its successful transference from a donor to a receptor muscle by a procedure similar to that already employed for transference on to a heart; but the probability of obtaining such transference will be very much more remote than for the heart, since for the latter the transferred effect may often much exceed in magnitude that which simultaneously appears in the donor muscle, *cf.* fig. 7.

The occasional appearance of a muscle capable of responding just perceptibly to an adrenaline concentration of 1 : 200 million has been recorded in § 2. The occurrence of donor preparations able to yield a perfusate having an adrenaline equivalent 1 : 200 million, as estimated on a heart preparation, has also been recorded (§4). Hence, by coupling a very potent donor with a highly sensitive receptor muscle, successful transference might be obtained; indeed, if the substance yielded by the donor is adrenaline, then a transferred effect must occur; but unless more potent donors or more sensitive receptor muscles exist than have been encountered in the present experiments, the effect obtained will only just be perceptible.

The procedure consists in setting up a succession of perfused ventral-root sartorius preparations till one is encountered capable of responding to adrenaline 1 : 200 million perfused through it. This is then retained as the receptor muscle. Ventral-root stimulation is stopped and a gentle perfusion maintained in the hope that the preparation will not deteriorate before a potent donor has been obtained. The donor is the usual perfused double sympathetic preparation. The capillary cannula, which is inserted into the abdominal vein for collecting the perfusate as it emerges from the limb muscles, is about 10 inches long, bent at right angles near one end to permit of its insertion down to the tapering end of the cannula perfusing the receptor preparation. The potency of the donor is now tested on a heart that is kept in readiness for the purpose. If suitable, the donor preparation is bodily removed and placed on a platform about 8 inches above the receptor preparation. Finally, the long capillary cannula from the abdominal vein of the donor is gently lowered into the cannula through which the receptor is perfused, till it comes to rest where the latter tapers off into the capillary which is inserted along the aorta of the receptor. A constant rate of perfusion of donor and receptor is maintained by keeping the head of Ringer in the two aorta cannulæ at

a constant level. Ventral-root stimulation of the receptor muscle, which has now much recovered, is recommenced. At various intervals the sympathetics of the donor are given prolonged stimulation, and the effect on the receptor muscle observed.

In fig. 8, Plate 19, is given a record from such an experiment. The potency of the donor as tested on the heart preparation is shown in fig. 8A; it is evident that sympathetic stimulation of the donor produces an increase in amplitude of beat rather greater than that given by adrenaline 1 : 200 million. The sensitiveness of the receptor muscle was tested both before and at the end of the experiment; the second of these records is shown in fig. 8B. Adrenaline 1 : 100 million yields a weak response; to 1 : 200 million it is just perceptible. In fig. 8C is shown the effect obtained on the receptor muscle when the sympathetics of the donor are stimulated. For three successive stimulations of the sympathetic there occurs a faint effect on the receptor muscle slightly greater than that given by 1 : 200 million adrenaline. There is a latent period of from 1 to 2 minutes; the whole effect has a duration of about 5 minutes. The record is reproduced here as a negative of the original, in which form the effect is more readily seen ; but even at best close scrutiny is necessary to detect it. The third response is shown with only slight reduction in fig. 8D.

A stronger effect would have been desirable for convincing demonstration; but unless more sensitive receptors, or more potent donors, occur than have been encountered in the present experiments, it is unlikely that this will be possible. A few attempts to sensitize the receptors with cocaine or glycocoll failed.

Experiments performed on less sensitive muscles were without effect.

§ 7—Interpretation of Orbeli's Effect

Consider now the character of the Orbeli phenomenon as a response to an adrenaline-like substance released by the sympathetic nerves around the larger blood vessels. The latent period—varying from 10 seconds for powerful responses to as much as a minute for very feeble effects—will be the time required for the substance, diffusing through the tissue spaces, to attain to effective concentration within a small number of fatigued or end organs, the maximum number not being affected till $\frac{1}{2}$ to $1\frac{1}{2}$ minutes, fig. 1, after cessation of stimulation.

The cause of the subsequent decline requires investigation.

(i) It is possible that the tissue becomes temporarily refractory to further molecules of the intermediary replacing those that are destroyed

in the motor end-organ. As a main cause of the decline this possibility is excluded by the fact that the tissue can respond, even additively, to a second stimulus inserted either at the onset or later in the period of decline, fig. 9. It is also excluded by the fact that an adrenaline response equal in magnitude to a particular Orbeli effect may be much more prolonged if the adrenaline be applied sufficiently long; for instance, in fig. 2 the response to 1 : 100 million adrenaline is better maintained than the slightly stronger effect due to sympathetic stimulation.

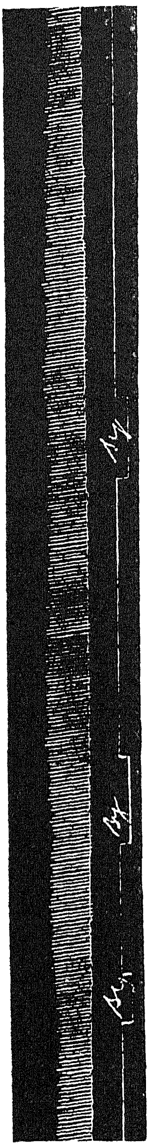

Fig. 9—Three responses to sympathetic stimulation, the second and third inserted early and late respectively in the phase of decline from a foregoing response. Sartorius; perfused; ventral-root stimulation. 15 per minute. 18° C.

(ii) The intermediary may undergo destruction in the muscle. According to Läwen (1903) adrenaline is partially destroyed in passage through the blood vessels of a frog; but the fact that the intermediary is detectable in the perfusate shows that this is not the sole cause of the decline.

(iii) The third possibility is that the chemical intermediary becomes diluted owing to dispersal through the muscle. That this is a potent factor in the decline is shown by the effect of perfusion.

This has been examined in the sartorius preparation, the perfusion being stopped when required by removing most of the fluid from the cannula. A record from an experiment is shown in fig. 10. During the first and third responses, the muscle is undergoing active perfusion; for the second and fourth the perfusion is stopped. In each case the sympathetic is stimulated for one minute. Simple inspection suffices to show that the decline is markedly steeper for the perfused than for the unperfused muscle. It may be suspected that the efficiency of the sympathetic becomes impaired by prolonged stoppage of perfusion, the succeeding response, with active perfusion, being therefore weaker. This possibility is excluded by an experiment of which fig. 11 is a record, three successive responses with perfusion being compared with three later responses with perfusion stopped. The immediate change in the character of the response with stoppage of perfusion is obvious, the rate of decline being visibly

less, even for responses of equal size (third and fourth), and even though the responses are diminishing as the sympathetic fatigues.

These observations, which have been made many times, seem to show that dispersal of the intermediary is the main cause of the decline, for dispersal through the muscle will be hastened by an active perfusion of its vessels.

§ 8—Nature of the Chemical Intermediary

Whilst the nature of the chemical intermediary must remain for the present undetermined, its relationship to, if not identity with, adrenaline is obviously suggested.

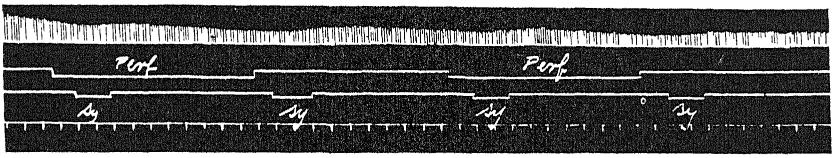

Fig. 10—Four successive responses to sympathetic stimulation, showing the effect of perfusion on the form of the response. Note that the effects are better maintained in the absence of perfusion. Sartorius. Time in half-minutes. 18° C.

The experiment involving transference on to another muscle offers particular information on this point. It will be evident that (i) the cardio-augmentor substance must be identical with the substance producing the transferred effect in another muscle, for the presence of two different substances in equal quantity (when measured in terms of adrenaline) is improbable; (ii) the activity of this substance, relative to that of adrenaline, is the same for heart and muscle. This argues for its identity with adrenaline, for it is unlikely that the activity of distinct sympathicomimetic substances relative to adrenaline should be the same when tested on various organs. For instance, tyramine, when tested on the frog's heart, is usually 100 to 400 times less active than adrenaline (in one exceptional case it was about 2000 times less active); yet it has no effect on muscle except at high concentration (1 : 5000) when its action is injurious. (All the muscles tested responded to at least 1 : 10 million adrenaline.) The action of ephedrine was also examined, but it had no effect on heart or muscle, except to act as a depressant at high concentration (1 : 1000).

That the substance should be identical with " sympathin " seems very probable. The similarity between the action of sympathin and adrenaline has been shown by Cannon and his co-workers. Recent work by Cannon

and Rosenblueth (1933) shows, however, that the parallelism is not complete, the intermediary apparently becoming modified according to its action (excitation or inhibition) in the tissues affected.

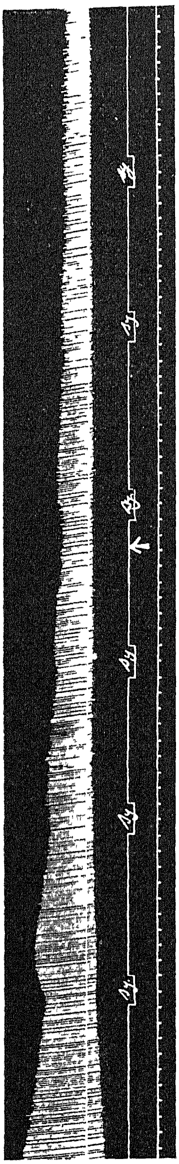

FIG. 11—Six successive responses to sympathetic stimulation, the first three with perfusion; to right of arrow perfusion stopped. Note that the effects are better maintained in absence of perfusion. Sartorius. Time in half-minutes. 17° C.

The identification of the intermediary by chemical means is uncertain. According to Bacq (1933) it is a catechol derivative.

Attempts to identify the cardio-augmentor substance by the fluorescence test, in the form recommended by Gaddum and Schild (1933), were without success. The pale green fluorescence, if viewed in a dark chamber in a strong beam of ultra-violet light, is distinguishable from the bluish tint of the alkaline Ringer control even at a dilution of 1 : 400 million; but adrenaline added to the perfusate from the hind-limb preparation is not recognizable at a dilution higher than about 1 : 10–20 million, being obscured by a pale fluorescence of the perfusate itself.*

§ 9—Discussion

The foregoing experiments provide evidence that the effects exerted by sympathetic nerves on skeletal muscle, so far as they consist of a reinforcement in the strength of contraction of the fatiguing muscle, are due to the release of a substance which acts as intermediary between the sympathetic nerves and the effect or tissue. Such chemical transmission, while not conflicting with the view of a direct sympathetic innervation of the muscle tissue, removes the only convincing physiological evidence which we have for it.

Since the sympathetic nerves of a muscle seem to occur exclusively in association with its blood vessels, especially the arterioles, it is here that the chemical intermediary will be discharged. The time relations of the muscular response are in good agree-

* I am indebted to Professor E. J. Hartung for kindly allowing me to do these experiments in his laboratory.

ment with this view. One cannot but suspect, therefore, that the chemical intermediary, particularly since it has an adrenaline-like action, will at the same time be a vaso-motor substance. This view presents certain difficulties.

(i) Although in the present experiments the vascular phenomena accompanying sympathetic stimulation have not been systematically examined, in the few experiments where they were sought they were not found, although good muscle effects occurred. On the other hand, in the experiments of Baetjer (1930) constriction of the vessels of the muscle occurred, thereby undoing the beneficial effect exerted on the muscle. This antagonism between the direct beneficial effect of sympathetic stimulation and the indirect injurious effect on vaso-constriction may seem to point to a separate sympathetic supply for blood vessel and muscle; but it is more likely that the discrepancy will disappear when the phenomenon has been examined under conditions of increased pressure, and redistribution of blood, that accompanies widespread sympathetic stimulation (Cannon).

In this connection the known facts concerning the action of adrenaline are suggestive. In the cat strong adrenaline contracts the blood vessels of muscle (Gunning, 1917; Gruber, 1929); but a weaker dose, capable of causing vaso-constriction in the skin, produces active dilatation in muscle (Hoskins, Gunning, and Berry, 1916; Dale and Richards, 1918) quite independent of any pressor effects (Gruber, 1929; Clark, 1934), though the latter when they occur enhance it (Gruber).

(ii) The experiments of Lewis and Marvin (1927) afford evidence against the release of a vaso-constrictor substance with adrenaline-like properties; for there is no delayed vaso-constrictor effect of sympathetic stimulation when the possibility of its removal from the tissue by an active blood flow is prevented by arresting the circulation. But it is not impossible that a vaso-constrictor substance, acting mainly on the arterioles, might undergo dispersal by diffusing into the tissue spaces. The fact that adrenaline, introduced directly into the tissue, can long hold up the circulation, would not seem to be an objection to this view, since a massive injection would not simulate the localized release of such a substance on the walls of the arterioles.

It is evident that for a clear understanding of the Orbeli phenomenon the attendant vascular effects require examination; and a renewed histological investigation into the possibility of a direct supply of sympathetic fibres to the muscle tissue seems desirable.

In conclusion, I have much pleasure in acknowledging my indebtedness to Mr. A. Doutch and Mr. H. Ennor for technical assistance, and to Professor J. T. Wilson, F.R.S., for reading the proofs.

10—Summary

When the sympathetic nerves to the skinned hind limbs of the frog are stimulated there is set free a substance which can increase the strength of contraction of (i) an isolated heart, (ii) another muscle into which it is perfused. It appears to be the substance whose release in muscle causes the Orbeli phenomenon. Its relation to adrenaline is discussed.

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12—Description of Plates

Plate 19

Fig. 4—Graded responses of a heart to adrenaline at concentration of 1 : 100 million, 1 : 500 million, 1 : 1000 million, 1 : 2000 million respectively. Time in half-minutes. Temperature 13·5° C. Original reduced to one-half.

Fig. 8—Transference of Orbeli's effect from a donor to a receptor muscle. A. Potency of donor, estimated by the heart preparation; at ↑ donor is disconnected; adrenaline 1 : 300 million without effect; adrenaline 1 : 200 million gives an effect rather smaller than that due to sympathetic stimulation. B. Response of receptor muscle to (i) 1 : 200 million adrenaline, (ii) 1 : 100 million adrenaline. C. Response of receptor muscle to sympathetic stimulation of donor. C. and D. shown as negative of original, and reduced to one-third. D. The third response of C. reduced to about two-thirds. Time in half-minutes. Temperature 18° C.

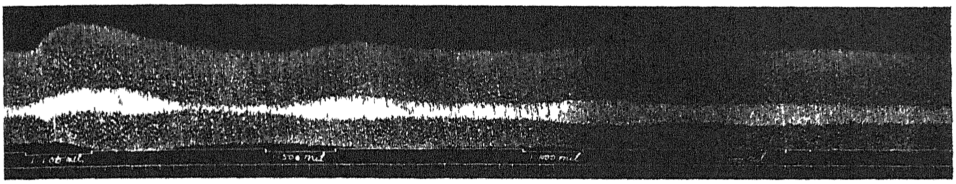

FIG. 4

A

B

C

D

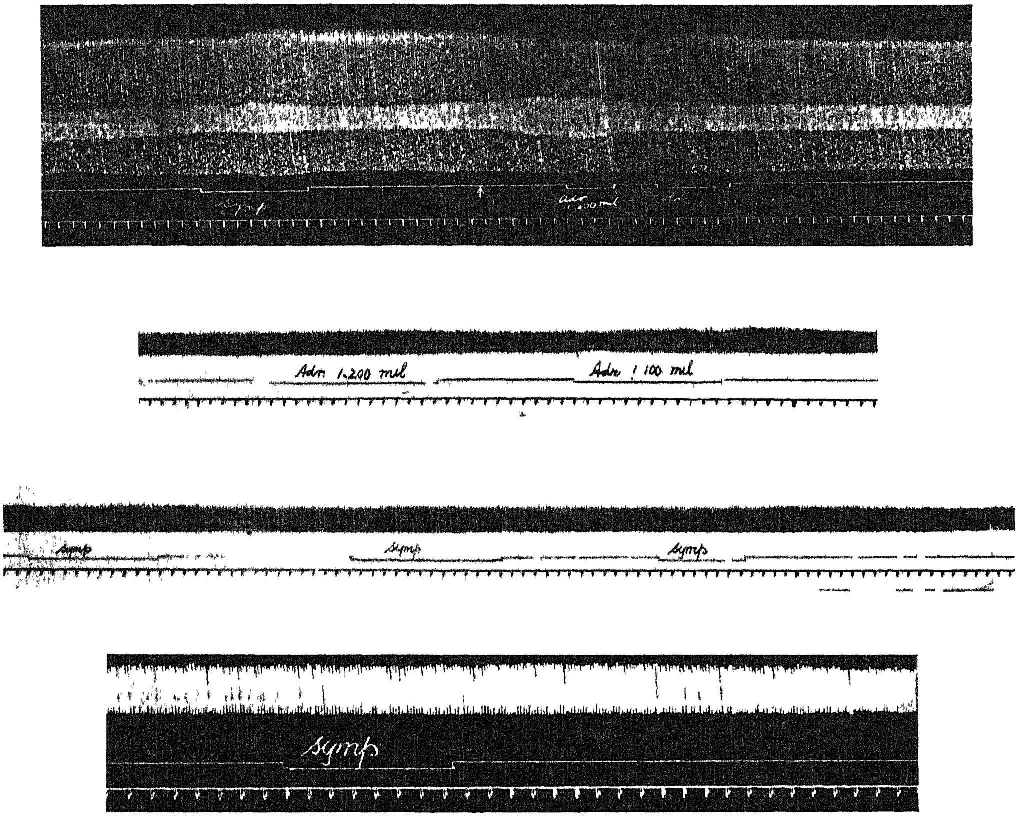

FIG. 8

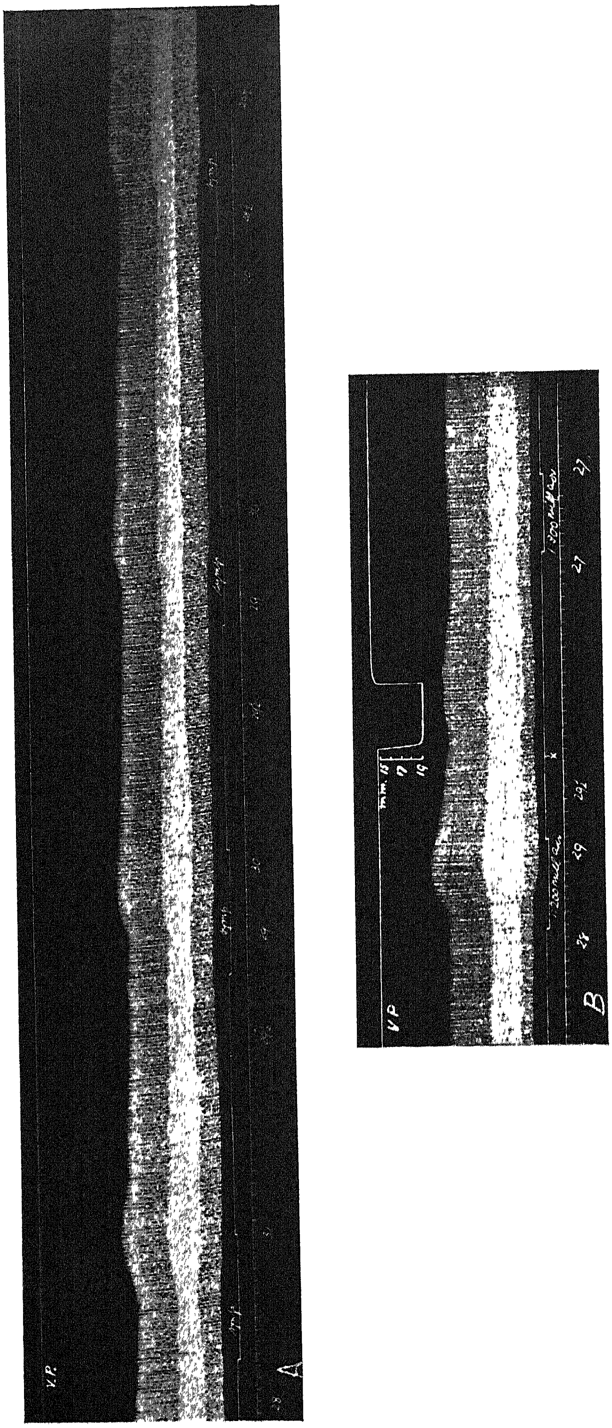

Fig. 5

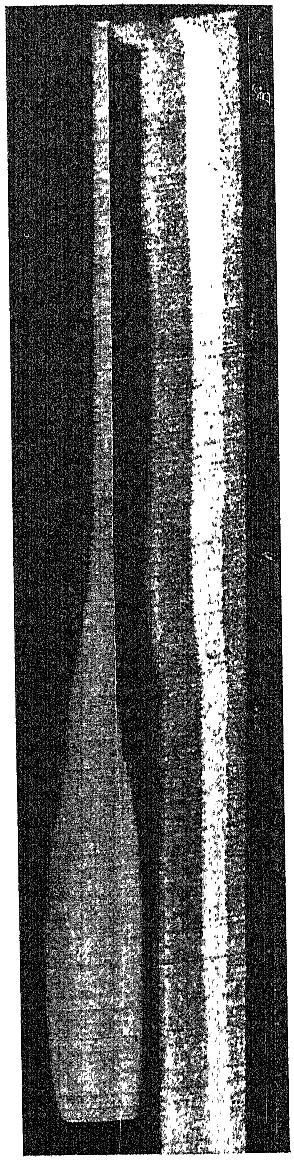

Fig. 7

PLATE 20

FIG. 5—A. Response of receptor heart to sympathetic stimulation of donor preparation. B. Response of same heart (donor removed) respectively to (i) 1 : 200 million adrenaline, (ii) increase in venous pressure by 4 mm at X, (iii) 1 : 300 million adrenaline. Upper line (V.P.) "venous pressure." Time in half-minutes. Frequency of heart beat per minute indicated below time marker. Temperature 15° C. Original reduced to one-half.

FIG. 7—Dissociation of Orbeli response from transferred heart effect by ergotoxine. Upper record—sartorius of donor; maximal ventral root stimulation; 15 per minute. Lower record—receptor heart. At A ergotoxine 1 : 50,000 perfused through donor into heart. At B. ergotoxine 1 : 50,000 + 1 : 1 million adrenaline perfused through donor into heart. The record of the final heart response is incomplete owing to heart lever striking against muscle lever. Time in half-minutes. Temperature 21° C. Original reduced to one-half.

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Studies on a Virus Causing Foliar Necrosis of the Potato

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(*Communicated by F. T. Brooks, F.R.S.—Received September* 17, 1934)

[PLATES 21 AND 22]

INTRODUCTION

In a previous communication (Bawden, 1932) two types of necrotic disease of the potato were described under the names of Acronecrosis (Top-necrosis) and Acropetal necrosis, names first suggested by Quanjer (1931). It was there shown that the external and internal symptoms of virus-infected potatoes are closely correlated, and that the former might with advantage be used as the basis of classification, instead of the latter, which are used by Quanjer. Further work has now shown that "streak" diseases of the potato exist, which cannot be included in either of the above groups, and which, in Quanjer's classification based on the internal symptoms of the stems, would have to be placed in the Anecrotic group. It is the purpose of this paper to describe some of the properties and reactions of a virus which in certain potato varieties causes a severe necrotic disease of the foliage without, apparently, producing any internal changes in the stem. It is proposed to call this disease Foliar necrosis.

The following features of Foliar necrosis serve with comparative ease to distinguish it from other necrotic diseases of the potato. Firstly, from Top-necrosis it is distinguished by the direction of spread of the symptoms (in Foliar necrosis it is acropetal, in Top-necrosis basipetal); by the presence of a pronounced mosaic, and also by the freedom of the main and axillary growing points from destructive necroses. Secondly, it is distinguished from Acropetal necrosis by the essentially interveinal character of the lesions, both mottle and necroses, and by the absence of the elongated brown streaks on veins, petioles, and stems so characteristic of this last disease. Thirdly, it is distinguished from both Top-necrosis and Acropetal necrosis by the absence of any internal lesions in the stems and by the two distinct phases of the disease which follow each other so rapidly in the first year of infection.

The virus causing Foliar necrosis was first obtained by Dr. R. N. Salaman from Professor P. Murphy in 1927 under the name of President Streak. There would seem to be little reason to think that the culture contains more than one virus. Infections have been carried out in various ways; the virus has been carried through many different plants, filtered and subjected to physical and chemical tests without markedly affecting its reactions. In common with some other potato viruses, however, the symptoms of disease caused by this virus depend greatly on the potato variety infected. In many varieties it causes Foliar necrosis, but in others it causes different diseases, whilst in yet others it is carried. In view of this, it would be confusing to give this virus a " symptom name," and for simplicity and convenience the alphabetical nomenclature, instituted for potato viruses by Smith (1931), will be followed. It is proposed to call the virus causing Foliar necrosis, potato virus " D."

Throughout the paper the symptoms described, unless otherwise stated, refer to those exhibited by plants grown and infected in an insect-proof glasshouse, with an average daily mean temperature of 23° C.

It has been found that much more accurate records of the necroses occurring on potato leaves can be obtained by photographing on infra-red plates, and some of the illustrations used in this paper have been obtained in this manner. In such photographs the normal leaf tissue appears white, and the necrotic areas are black.

Virus " D " and Foliar Necrosis

Arran Victory—The following description is based on a large number of infections of Arran Victory plants with virus " D," made both by

needle inoculations and stem grafts. The systemic symptoms obtained are identical, whichever method of infection is employed, but with needle inoculation local lesions are frequently produced at the seat of inoculation. These local lesions usually appear about 8 to 10 days after inoculation, but they are not invariably produced. They are most readily and abundantly formed when inoculation is carried out in such a manner that the leaf hairs are broken whilst the epidermis is not materially damaged. Excessive damage to the leaf tissue may inhibit the production of local lesions without preventing infection. The local lesions appear externally as black necrotic spots which penetrate the thickness of the leaf, fig. 1, Plate 21. They are usually more or less circular, brittle to the touch, and after a time tend to fall from the surrounding leaf tissue to give a "shot-hole" appearance. They are extremely localized, and once formed show little tendency to spread.

The first systemic symptoms usually appear about 17 to 19 days after infection, and consist of interveinal necrotic blotches on the under surfaces of those leaves occupying an intermediate position on the stem, fig. 2, Plate 21. These necroses are greyish in colour and are best seen by transmitted light. They are soft and damp to the touch, and give the leaf a wilted appearance. The necroses spread rapidly, coalesce and ultimately affect the whole leaf, so causing it to wilt and finally fall, fig. 3, Plate 21. The advance of the disease is acropetal, and, starting with the intermediate leaves, the wilting and falling of the leaves spreads upwards. The extent of the wilting and falling depends somewhat on the environmental conditions and on the stage of growth of the plant. At comparatively high temperatures all leaves except the very uppermost are affected and may fall, so that a "palm tree" effect is obtained in which all the lower leaves are fallen, and a bare stem is left surmounted by a crown of leaves, fig. 4, Plate 21. At lower temperatures, and more especially with older plants, the wilting and falling are confined to the intermediate leaves which first show systemic necroses. The apical and axillary growing points are never killed.

These primary symptoms are then followed by a secondary and less severe stage of the disease. The upper leaves show a pronounced and rather blotchy interveinal mosaic. In addition, spotty black interveinal necroses appear which may cause acute local deformity. These necroses differ widely from those of the primary stage of the disease, and more closely resemble the local lesions, fig. 5, Plate 22. They are hard, black, and brittle, show little tendency to coalesce and remain as isolated patches of dead cells penetrating the thickness of the leaf. With the secondary stage of the disease the wilting and falling of the leaves are arrested;

any new growth from such plants shows only the interveinal mottle and the scattered black necroses.

The tubers obtained from plants suffering from Foliar necrosis are small but apparently healthy, and on planting sprout normally. Arran Victory plants in the second year of infection with potato virus "D" show symptoms very similar to those of first year infected plants in the secondary stage of the disease. Early in the season they exhibit a definite interveinal mottle and occasional scattered black interveinal necroses. As the plant matures the mottle tends to become less pronounced, whilst the necroses increase in severity. These may cause acute local deformity, and the disease picture presented is that of a crinkle plus necrosis. The diseased plants are much smaller and less well developed than healthy ones, and they mature earlier. There is, however, no wilting and falling of the leaves, so characteristic of first year infection.

In the field such plants are much dwarfed, mature very early and set only a few small tubers. The leaves are much underdeveloped, extremely harsh, and brittle, and covered with many small black necroses. The mottle is usually less well developed than on plants grown in the glass-house. In addition, the leaves are often definitely bronzed, the so-called "rust," a condition which seems to be peculiar to virus-infected plants grown in the open.

Histological examination of the leaves of plants suffering from Foliar necrosis shows that the necrotic process originates in the parenchymatous cells abutting on the small vascular bundles found between the main veins. These cells swell, their cytoplasm becomes abnormally granular and yellow, whilst the walls are thickened by deposits of suberin or cutin. The plastids rapidly degenerate, their breakdown often being the first obvious sign of disease. Cells some distance from demonstrably necrotic ones frequently show plastids in all stages of degeneration. Affected cells may show either of two types of reaction. In some, the whole cell contents appear to be used up in the thickening of the cell walls. In others, large deposits, apparently rich in tannin and pectin, are formed. These first make their appearance as light yellow granules; the granules coalesce, darken in colour, and in the dead cells are seen as brownish masses adhering to the walls. From the region of the small vascular bundles the necroses spread outwards to the epidermis and along the length of the leaf affecting both palisade cells and spongy parenchyma. In the primary stage of the disease the necroses spread from the parenchyma into the veins, affecting all the tissues equally, and from these pass down into the petioles. In the secondary stage, however, no internal changes are seen in the petioles. Examination of the stems and tubers of plants

suffering from Foliar necrosis has, as yet, failed to show the presence of any internal necroses or other abnormality. In acute cases, however, the " wing " of the stem is slightly necrosed.

Changes of another character are observed in the leaves owing to the occurrence of the mosaic symptoms. The mottled areas are noticeably thinner than the green, the palisade cells of the former being considerably underdeveloped. Also, the individual plastids are smaller and stain much less deeply than do those in normal cells. An extensive search for intracellular inclusions, or " X " bodies, has not been made. If they occur, however, their occurrence must be rare, for none has been seen in a large number of preparations made from plants known to be infected with virus " D " alone.

President—The disease induced by potato virus " D " on President plants differs only slightly from that described for Arran Victory. The primary stage of the disease is identical; wilt-like necroses appear on the intermediate leaves which shrivel and fall, and the disease spreads acropetally. In the secondary stage, however, the interveinal mottle is usually more pronounced than in Arran Victory and the necroses are fewer, although of a similar type. Similarly, in the second year of infection President plants are not usually so severely affected as Arran Victory. The interveinal mottle is much brighter, but the black necroses are fewer and do not cause such acute deformity.

The flowering of this variety seems to be affected by infection with potato virus " D," and very few flowers, if any, per plant are produced. When obtained, these flowers have been tested for the presence of the virus, and it has been found in calyx, corolla, andrœcium, and gynœcium. Up to the present it has proved impossible to obtain any pollen from infected plants, and it is not known if this would contain the virus.

Other Potato Varieties—In addition to Arran Victory and President the following potato varieties have been found to show the symptoms of Foliar necrosis when infected with virus " D ":—Arran Cairn (2), Arran Chief (4), Arran Comrade (6), Arran Pilot (2), British Queen (5) Edzell Blue (3), Katahdin (2), Kerr's Pink (8), May Queen (5), Rhoderick Dhu (4), and Sharpe's Express (4) (the figure in parentheses after each variety refers to the number of individuals of that variety infected). In all these varieties the disease passes through the two typical phases, and the first symptoms consist of a necrosis of the middle leaves, which later wilt and fall. In the secondary stage of the disease the lesions in all are of a similar nature, but the proportion of mosaic to necrotic symptoms varies somewhat from variety to variety.

Virus " D " and Top-necrosis

In certain potato varieties infection with virus " D " brings about Top-necrosis, a disease which in different varieties may be caused by different viruses. In Epicure, Arran Crest, and King Edward plants it causes a disease identical in symptoms with that induced by potato virus " X " (Salaman and Bawden, 1932). On grafting, the first symptom is an extensive necrotic spotting of the uppermost leaves surrounding the growing point. The necroses coalesce, kill out the growing point and the plants die back from the top. Internal lesions are found in the stems of such plants. These originate in the phloem, most frequently in the internal phloem, and spread from there to all other tissues. Tubers set by these plants look normal when harvested, but during storage some become necrotic and a few are killed. When planted such tubers either fail to grow or give rise to plants which never reach a height of more than a few inches, are covered with fine necroses and quickly die. Tubers which do not develop necroses during storage seem to have escaped infection, possibly owing to the too rapid death of the stolons, and on planting give rise to healthy plants. Four Epicure and Arran Crest plants and three King Edward have been grafted, and all have given this result. When needle-inoculated with virus " D " these varieties usually show severe local lesions, and occasionally no systemic symptoms are obtained. Eight Epicure plants, five Arran Crest, and six King Edward have been inoculated and four, three, and four respectively developed Top-necrosis, whilst the remainder showed only local lesions. It would seem possible in such cases that the action of the virus on entry is so lethal that the inoculated cells are killed, and the virus is prevented from entering further into the plants.

A type of Top-necrosis is also obtained when Arran Consul, Majestic, Up-to-Date, and Duke of York plants are infected with virus " D," but the external symptoms differ somewhat from those described above. The first necroses appear on the veins of the uppermost leaves or at the top of the main stem, and consist of dark brown or black stripes, fig. 6, Plate 22. From the veins the necroses pass into the parenchyma and the whole apex of the plant is killed. The progress of the disease now depends largely on the stage of growth of the plant. If young and fleshy it usually dies back from the top, and the whole plant is killed. If older and woody, the destructive necroses are frequently restricted to the actual growing points. In such plants the necroses cause acute twisting and crinkling of the uppermost leaves. Internal necroses are found in

the stems and petioles, and originate in the phloem. The bundles chiefly affected, however, in these varieties are those of the external phloem, and from these the necroses spread outwards through the cortex to the epidermis, appearing externally as black stripes, fig. 7, Plate 22. The tubers set by these plants develop necroses during storage, and when planted give rise to small, deformed plants, the veins and stems of which are acutely necrotic. Such plants quickly die without setting any fresh tubers. Five plants each of Majestic and Arran Consul have been grafted and inoculated with virus " D " and all have shown the symptoms described above. Five Up-to-Date and three Duke of York plants have been grafted and these have also shown this type of Top-necrosis in the new side shoots. When needle-inoculated, however, these varieties have given different results. Six Duke of York plants have been needle-inoculated and no results obtained. Of nine Up-to-Date plants inoculated six gave no result, and the other three gave the Top-necrosis. The symptoms, however, in all three were confined to the actual growing points, and did not spread down the stems. The stocks of these two varieties used are infected with a mild strain of potato virus " X," and the reasons underlying these results are discussed in the second part of the paper.

Eleven Abundance plants have been infected with virus " D " and showed Top-necrosis. The type of symptom, however, was again somewhat different from either that of Epicure or Majestic. Necroses first appear on the leaves immediately below the apex, and spread from these up into the growing point, which is then killed. The spread of the disease then becomes basipetal, and the plant dies back from the top. Internal lesions are found in the stem, where they originate in the phloem and pass out to the epidermis. The tubers set by infected Abundance plants have all developed necroses during storage, but have all sprouted on planting. The second year plants are much dwarfed, and are at first smothered with numerous small black necroses. These coalesce, killing out large areas of the leaves, which shrivel and fall, and the whole plant soon matures, fig. 8, Plate 22.

Carriers of Potato Virus " D "

Six plants of Arran Banner, two of Arran Scout, seven of Champion, four of Eclipse, and three of Di Vernon have been infected with virus " D " and have shown no symptoms. That they were infected was shown by grafting scions from them back on to Arran Victory or President plants, when Foliar necrosis was produced in all the stocks.

Great Scot and International Kidney plants may also behave as carriers under certain conditions. Twenty-one plants of these two varieties have been inoculated or grafted with virus " D " and 14 have shown no symptoms: although when tested they were found to be infected. Of the remaining seven plants each gave slight lesions, all of the same character. The middle leaves developed a certain amount of necrosis which spread only slowly, and the topmost leaves never showed any sign of disease, although infected with the virus. In the second year of infection none of the plants exhibited any disease symptoms. The reason for this variation in reaction is not known, but it was noticed that the necrosis on these varieties was more severe and more frequently produced when the plants were growing at high temperatures, and when they were injured or their growth checked in any way. Second inoculations of virus " D " to infected Great Scot and International Kidney plants have failed to produce any further reaction.

Virus " D " on Species other than the Potato

The host range of virus " D " extends beyond the potato and it has been transmitted to the tobacco, var. White Burley, *Nicotiana glutinosa*, the tomato and *Datura Stramonium*.

Tobacco plants, infected by lightly rubbing the leaves with infective juice, occasionally give local lesions. These are extremely mild and consist of a faint chlorotic spotting of the inoculated leaf, appearing 5 to 7 days after inoculation. Systemic symptoms are obtained 12 or 13 days after infection, and first appear as chlorotic spots on an intermediate leaf. The chloroses increase in size and frequently assume a ring form. The spots and rings are always most definite on the intermediate leaves, and the disease is never severe, fig. 9, Plate 22. As the tobacco grows the symptoms tend to fade and within 5 to 6 weeks after infection the plants often look quite healthy.

Virus " D " has been cultured continuously in tobacco for 3 years without producing any permanent change in its symptoms. Those obtained in the winter months when the light intensity is low are always rather more severe than those obtained in the summer. This variation, however, is only temporary, for a culture of the virus, giving a bright yellow ring mottle in the winter, reproduces only the typical mild disease in the following summer.

On *Nicotiana glutinosa* the disease produced by virus " D " is similar to, but much less severe than, that on tobacco. It consists of an extremely faint chlorotic spotting of the intermediate leaves, which persists for

three weeks to a month, after which the plant may look quite healthy.

On tomato, var. Kondine Red, it produces a very faint but general interveinal mosaic. When such tomato plants are further infected with the virus of tobacco mosaic "experimental," or "glasshouse" streak is produced.

The reactions of *Datura Stramonium* to infection with virus "D" have shown some variation. Some three hundred plants have been infected and over two hundred have shown no symptoms, and have proved to be carriers of the virus. The remainder have shown faint but fairly characteristic symptoms. These have usually appeared about 10 to 12 days after inoculation, and consisted of a mild chlorotic spotting, fig. 10, Plate 22. The spots persisted for about a fortnight, after which they faded, and the plants again looked healthy. The carrying power of *Datura Stramonium* has been further tested in the following manner: scions of healthy *Datura* were grafted on to four President stocks and allowed to grow. After a fortnight virus "D" was needle-inoculated, in two plants to the stocks and in the other two to the scions. In all four the President stocks developed typical Foliar necrosis, whilst the *Datura* scions remained quiet healthy in appearance. There would thus seem to be little doubt that under certain conditions *Datura* can carry virus "D," but that the carrying power can be upset. It has been noticed that infected *Datura* are more prone to show symptoms when growing under poor light conditions, and that under these conditions the symptoms are more definite. Early in the work it was extremely rare to find any symptoms on *Datura*, but with continuous culture of the virus in tobacco and *Datura* they have become more frequent and more pronounced, in some recent infections amounting almost to a mild interveinal mottle and green veinbanding.

Purification of Potato Virus "D"

Attempts have been made to free virus "D" from the accompanying plant protein in two ways. The method used by Vinson (1932) for the virus of Tobacco mosaic, in which the virus is first precipitated by safranin and subsequently eluted with Lloyd's reagent, has always given negative results, the water-clear final product being non-infectious. The reason for the failure of this method seems to be that only a very small proportion of the virus is precipitated by safranin, and this small proportion is itself precipitated by the Lloyd's reagent.

The method used by MacClement (1934) for the purification of potato virus "X" has, however, been applied with some success. The expressed sap is diluted 15 times with distilled water, cooled to 0° C and saturated with carbon dioxide. After centrifuging, the supernatant liquid is diluted at least 10 times with distilled water at 35° C, and saturated with CO_2 at this temperature. After centrifuging, the precipitate, which is small and thrown down only after a long period, is shaken up in distilled water equal in volume to the original expressed juice. This suspension is again centrifuged, and the supernatant contains the pure virus. This is usually slightly opalescent, but sometimes is quite water clear. The opalescence, however, can always be removed by filtration through coarse membranes,* without appreciably affecting the virus content, when a water-clear fluid is obtained.

Virus suspensions prepared this way vary as to their virus content, as measured by the dilution end-point. Such suspensions are rarely infectious at dilutions greater than 1 in 300, and occasionally fail to infect plants at a dilution of 1 in 100. Virus appears to be lost at two stages of the process: in the first and third precipitates, which are discarded. The amounts lost can to some extent be controlled by keeping the temperature as near 0° C as possible during the first centrifuging, and by making the time of the third as short as possible. The amount of virus which can be recovered from these two precipitates does not, however, satisfactorily account for the great difference between the dilution end-points of the crude expressed sap and the purified suspension. It is possible that a proportion of the virus is actually destroyed during the process, but as the temperature never exceeds 35° C it is difficult to believe that this can be large. A more probable explanation is that in removing the adsorbing plant protein the attraction of the actual virus particles for each other is increased and they tend to aggregate. If this be so, it is obvious that the dilution end-points of the crude sap and purified virus cannot be used as comparative indicators of their respective virus content. Attempts to prove that this aggregation is occurring have failed. The pure virus has been subjected to different p_H values in an attempt to break up possible aggregates of virus particles, but this apparently has only resulted in causing further aggregation. Samples of the pure virus infectious at 1 in 250 at p_H 7 failed to give infections at p_H 5·5 or p_H 8·5 at a dilution greater than 1 in 10. Thus it would seem that, if aggregation is occurring, it is less when neutral than when either acid or alkaline.

* The filtration was performed by Dr. K. M. Smith, to whom thanks are due.

The method appears to be independent of the host plant, and has been used with equal success for purifying virus obtained from infected tobacco, potato, and *Datura Stramonium* plants.

Attempts to Transmit Virus " D " by Insects

As yet, no successful transmission of virus " D " has been obtained with any insect. The following have been tested as possible vectors:—*Myzus persicæ* Sulz., *M. circumflexus* Buckt., *M. pseudosolani* Theob., *Macrosiphum gei* Koch., *Aphis rhamni* Boyer, *Eupteryx auratus* Liv., *Thrips tabaci* Lind, and *Lygus pratensis* Linn. Transmissions have been attempted from potato to potato and tobacco, and from tobacco to tobacco and potato, and all have given negative results.

Physical and Chemical Properties of Virus " D "

(*a*) *Filterability*—Experiments on the filterability of virus " D " were made with juice derived from infected potato and tobacco plants and similar results obtained. A preliminary filtration to clarify the sap was made through either sand and pulp, or a kieselguhr bed. The clarified sap was then filtered through Chamberland candles L 1, L 3, and L 5. The filtrates from all were infectious, and on inoculation to Arran Victory produced Foliar necrosis and to tobacco a mild chlorotic mottle, but the virus content of the L 5 filtrates was low.

Table I

Temperature °C	Plants inoculated	Plants infected	
60	30	29	97%
63	12	10	83%
64	12	9	75%
65	18	9	50%
66	18	4	22%
67	30	2	7%
68	18	0	0%
70	30	0	0%

(*b*) *Effect of Heat*—1 cc samples of juice from infected plants were heated for 10 minutes in thin walled glass tubes in a water bath kept constant at various temperatures. After cooling rapidly, the juice was immediately inoculated into batches of six tobacco plants. The summarized results of six such experiments with sap derived from infected tobacco plants are given in Table I.

It will be seen that the thermal death point, *i.e.*, the temperature at which the virus is completely inactivated, under these conditions is about 67° C, but that a considerable falling off in the infective power is shown some degrees below this. It thus appears that 67° C represents the thermal death point of only the most heat resistant fraction of the virus, and that actually for a random sample a thermal death range of some few degrees exists. This was further tested in the following manner. Infective tobacco juice was heated for 10 minutes at 60° C. Six plants inoculated with this juice undiluted all became infected. When diluted, however, no infections were obtained at a dilution greater than 1 in 100, whereas the unheated control gave 100% infection at a dilution of 1 in 1000. A part of this fall in dilution end-point may be due to the precipitation of the virus by the coagulation of the plant protein, but this can hardly account for all of it. It would, therefore, seem probable that the thermal death range is even greater than can be detected by inoculation of the undiluted sap, and extends from below 60° C to 67° C. Continuous heating for 1 hour at 60° C failed to inactivate the virus completely.

Infective potato sap and suspensions of the purified virus were also heated. These were found to be infectious after 10 minutes at 65° C (4 plants out of 6 infected with the potato sap and 2 out of 6 with the pure virus), but were completely inactivated at 67° C. The thermal death point, therefore, seems to be unaffected by the presence of plant protein, and to be independent of the host plant.

(*c*) *Longevity* in vitro—The length of the survival of virus "D" *in vitro* has been determined in expressed juice from potato and tobacco, in filtered sap and in suspensions of the purified virus. The results obtained from these have all been similar, but with each it has been found that the longevity of the virus depends greatly on the temperature at which it is kept. At a temperature of 25° C the infectivity of the virus rapidly decreases and it is completely inactivated in 6 or 7 days. At 10° C the virus was still infective after 12 days' storage, and at 1° C it has been found to be infectious after a month, but not after 6 weeks.

(*d*) *Effect of Alcohol, Phenol, Formalin, Saponin, and Psychosyn*—The strengths of the solutions as given below refer to the final strength after mixing with the virus juice, *e.g.*, 2% phenol indicates that 1 cc of 4% phenol was mixed with 1 cc of virus suspension. In all tests the mixtures were allowed to stand for 1 hour before inoculation to the test tobaccos. After inoculation the leaves were immediately washed with water to prevent any damage to the leaf tissue by the substance under test. The

tests have been conducted on expressed juice from infected tobacco plants and on suspensions of the purified virus, with no significantly different results except with psychosyn.

It has been found that the virus is completely inactivated by 60% alcohol acting for 1 hour, but not by 50%. By phenol and formalin it was destroyed by solutions of 4% but not by 2%, also acting for 1 hour. Saponin at 1 in 20 was found to have no effect on the infectivity. Psychosyn, a lytic principle obtained from brain tissue, at 1 in 20, inactivated the purified virus but had no effect on the infectivity of crude expressed sap. When added to the latter an immediate precipitate was produced and it seems that all the psychosyn was used up in inducing this, leaving none to affect the virus.

(*e*) *Effect of Dilution*—The dilution end-point of sap derived from plants infected with virus " D " has been found to show some considerable variation. It seems to depend both on the host plant from which the sap is derived and on the state of growth of the plant at the time. With juice derived from young actively growing tobacco plants infections are usually obtained at a dilution of 1 in 5000, but not at 1 in 10,000. From old tobacco plants the effect of dilution is much more marked and the dilution end-point is usually in the region of 1 in 1500. With sap derived from potatoes no infections have been obtained at dilutions greater than 1 in 2000, and at this dilution only when the plant was actively growing. From more mature plants the dilution end-point was frequently found to be as low as 1 in 750.

Experiments on Immunity and Resistance

In inoculating virus " D " to potatoes it was noticed that considerable difficulty was experienced in infecting certain varieties which were known to be already infected with potato virus " X." It has previously been shown by Salaman (1933) that virus " X " can exist in different strains of virulence, and that the presence of a mild strain in a plant protects it against further infection by a more virulent one. It was thought possible that some such protective effect was being afforded to plants infected with virus " X " against further infection with virus " D," and experiments were therefore set up to test this. As *Datura Stramonium* reacts characteristically when infected with virus " X " and gives very slight, or no, symptoms with virus " D " it was first chosen as a suitable plant with which to work.

Batches of *Datura* plants of equal size and age were divided into three sets. Two sets were inoculated with virus " D," and the third left

untouched to act as control. Fourteen days later, all the plants still being quite healthy in appearance, one of the sets inoculated with virus "D" and the hitherto untouched one were inoculated with a virulent strain of the "X" virus. Five days after this reinoculation the controls showed severe local lesions consisting of white necrotic double rings, whilst the plants previously infected with virus "D" did not. Similarly, eight days after the second inoculation the controls showed severe systemic symptoms, consisting of acute necroses, bright interveinal mottle and leaf deformity, whilst both sets of plants inoculated with virus "D" were quite healthy in appearance, figs. 11 and 12, Plate 22, the further inoculation of virus "X" to the one set having failed to produce any new symptoms. This experiment has been repeated many times. Altogether over 100 *Datura* plants infected with virus "D" have been reinoculated with virus "X," with the same result, the *Daturas* infected with virus "D" being apparently completely resistant to the effects of the "X" virus. Occasionally *Datura* used for reinoculation have shown the faint symptoms sometimes induced by virus "D." No increase in severity of symptoms has followed on any reinoculation, the reinoculated plants showing symptoms identical with those of the controls infected with "D" alone, and usually the plants have later become quite healthy in appearance.

The time required for the *Datura* inoculated with virus "D" to acquire immunity to virus "X" varies somewhat with the size of the plant. With small seedlings the protection is often complete 5 to 6 days after the first inoculation. With older plants the time required is longer, more especially to render the older leaves immune. When reinoculating the older leaves of large plants with virus "X" local lesions are frequently obtained as long as 10 days after infection with virus "D," but the necrotic lesions are confined to the inoculated leaves, and no systemic symptoms follow.

Ten plants of *Datura* infected with virus "D" have been reinoculated with a virulent strain of virus "X" on four successive occasions at intervals of 14 days and have shown no signs of any "X" infection. It seems, therefore, that the acquired resistance is a lasting one, and once established persists throughout the life of the plant.

Return inoculations have been made from *Datura* first infected with virus "D" and then reinoculated with virus "X," and these have all failed to show the presence of the latter in such plants. When made to further healthy *Datura* these remained healthy in appearance, and when made to tobacco these showed the chlorotic mottle characteristic of infection with virus "D" alone.

The type of " X " virus used in the reinoculation seems to be immaterial, and virus " D " has been found to protect against all equally. Virus " D " infected *Datura* have been reinoculated with Salaman's *s*, *l*, and *g* strains of virus " X " (1933), and have shown no symptoms typical of infection with any. Also, further sub-inoculations from these plants to other healthy *Datura* seedlings have always failed to give any reaction characteristic of these strains.

Tobacco, var. White Burley, infected with virus " D " has also been found to show a definite resistance to further infection with virus " X." In most experiments this resistance has been found to be complete and to amount to immunity ; after reinoculation with the " X " virus the plants showed lesions identical with those of the control plants infected with virus " D " alone. On sub-culture to healthy *Datura* these plants were found to be free from the " X " virus. Occasionally, however, reinoculated tobacco plants have shown a few scattered necrotic rings, similar in type, but considerably less in number and severity, to those shown by the controls infected with " X " alone. By using a technique of Dr. Salaman which will shortly be published, it was possible to isolate and punch out these necrotic rings from the surrounding tissues. These were then ground up and the sap from them inoculated to healthy tobacco, to *Datura*, and to Arran Victory potatoes. The tobacco and *Datura* seedlings reacted with typical " X " symptoms, but the potatoes did not develop Foliar necrosis and showed only an interveinal mottle. From this it would seem that the actual entry of virus " X " into " D " infected tobacco is not necessarily prevented, but that the two viruses are unable to multiply in the same tissues. If this is so, such areas as showed the necrotic rings and from which virus " D " could not then be recovered, are probably those in which virus " D " had not become completely systemic, and in which, therefore, " X " could multiply. In an attempt to test this theory further, tobacco plants, which had been infected with virus " D " for 6 weeks, were inoculated over their whole surface with a virulent culture of virus " X." After a lapse of 5 days several scattered necrotic rings appeared on the older leaves, fig. 13, Plate 22, but no later systemic " X " symptoms were obtained. These necrotic local lesions were then isolated and inoculated to tobaccos and Arran Victory potatoes. Foliar necrosis did not develop in the latter, and the tobacco showed a typical severe " X " reaction. Similar isolations were also made from the chlorotic tissue between the necrotic rings, and these were also inoculated to tobacco and Arran Victory potatoes. These gave no " X " reaction on the former and caused Foliar necrosis on the latter. It thus seems

fairly clear that the areas in which the " X " virus produced symptoms contained no virus " D."

Tobacco plants infected with virus " D " are protected only against infection with virus " X," and are still susceptible to further infection with other viruses. Such tobaccos have been successfully infected with the viruses of tobacco mosaic, tobacco ringspot, tomato spotted wilt, and potato virus " Y." When reinoculated with the virus of tobacco mosaic or virus " Y," diseases more severe than those caused by either virus acting alone are produced. With tobacco mosaic the plants become severely necrotic and in extreme cases are killed. With virus " Y " the veins become bright yellow, whilst the interveinal areas bulge to give the leaves a corrugated appearance, and the growth of the plants is considerably hampered.

Plants of *Nicotiana glutinosa* infected with virus " D " also show some degree of resistance to further infection with virus " X," but usually less than either *Datura* or tobacco. Occasionally the protection is quite complete; 18 plants infected with virus " D " were reinoculated with virus " X " and 5 of these showed no increase in disease symptoms. The remaining plants, however, all showed an increase in severity of the disease, and the faint chlorotic spotting produced by infection with " D " alone was augmented by many scattered necrotic spots and rings. In no plant was the resulting disease nearly so severe as that induced in the control plants infected with " X " alone. The two viruses tended to segregate out in these plants, and by isolating the necrotic and mottled areas and inoculating them separately into further healthy plants it was found possible to regain pure cultures of the " X " and " D " viruses respectively. It is possible that virus " D " becomes less completely systemic in *Nicotiana glutinosa*, and it may be because of this that the degree of protection afforded is less than in tobacco and *Datura*.

Six plants of Arran Victory suffering from Foliar necrosis have been inoculated with virus " X," derived from a President plant showing a mild interveinal mottle. These showed no change in symptoms and remained similar in appearance to the control plants infected with " D " alone. Sub-inoculations made from these plànts back to healthy tobacco and *Datura* seedlings gave only a mild chlorotic mottle on the former and nothing on the latter. It would thus appear that the plants were protected against infection with virus " X," as six healthy control plants inoculated at the same time were all infected.

Experiments have also been performed to determine whether the protection is reciprocal, and if infection with virus " X " renders plants resistant to further infection with virus " D." With plants of *Datura*

Stramonium this has been found to be so. *Datura* infected with virus "X" has been reinoculated with virus "D," and, as might be expected, no change in the disease picture occurred. Sap from these plants was then needle-inoculated to healthy Arran Victory and President potatoes, and these later developed a mild interveinal mottling and not Foliar necrosis. The protection has been further tested in the following manner: scions of *Datura* infected with virus "X" were grafted to three healthy Arran Victory stocks and allowed to grow. After a period of 4 weeks the scions were inoculated with virus "D." The Arran Victory stocks developed only an interveinal mottling, characteristic of infection with "X" alone.

With potatoes the reciprocal protection has been easier to test as the "X" virus induces only a mild interveinal mottling whilst "D" causes the characteristic Foliar necrosis. Ten Arran Victory and President plants, previously infected with virus "X" and showing a mild mottle, have been inoculated with virus "D." Of these, nine showed no change in symptoms, but one, after 20 days, developed a few scattered greyish necroses on the middle leaves, which began to wilt. The disease never became as severe as in the control plants, and after a while the plant showed only a mild mottle and a few scattered necroses. By isolating these necroses and inoculating sap from them to healthy Arran Victory plants typical Foliar necrosis was produced.

Five "X" infected Arran Victory and President plants have been grafted with scions infected with virus "D," and in each a necrotic disease was produced in the stocks. About 20 days after grafting, the leaves immediately below the apices of the new, rapidly growing side shoots became dotted with greyish necrotic areas. These did not spread and coalesce to the same extent as in the control plants infected with "D" alone, and were never sufficiently acute to bring about the falling of the leaves. The upper leaves showed a pronounced interveinal mottling and a few scattered black necroses, *i.e.*, the picture was that of a much reduced Foliar necrosis. Following this, four President plants infected with virus "X" were inoculated with virus "D" and at the same time their tops were cut off, and the plants were placed in the warm moist grafting box. The young side shoots grew rapidly and three of the plants developed fresh symptoms similar to those described above for the grafted plants. The fourth plant developed only a mottle, and inoculations taken from it to other potatoes gave no indication of the presence of virus "D."

It is apparent from the above experiments that potatoes infected with virus "X" are rendered resistant to subsequent infection with virus "D." The amount of the resistance, however, seems to vary and to

depend greatly on the rapidity of the growth of the plant at the time of the second inoculation. With the plants growing normally the resistance has been complete, but when they were rapidly producing new tissues definite infections have been obtained. When plants were infected with both viruses, however, the symptoms of the resulting disease were never so severe as those produced in the control plants infected with virus " D " alone.

Discussion

From the above experiments it seems clear that the resistance afforded to plants infected with virus " X " against infection with virus " D," or *vice versa*, is not absolute, but varies somewhat in different species, and is also dependent to some extent on the rate of growth of the plant. It also appears that the actual entry of the second virus is not necessarily prevented, but rather that the two viruses are unable to multiply in the same tissue. This seems to imply that the two viruses have similar metabolic processes, each possibly affecting the same materials in the cell, as has also been suggested by Kunkel (1934) for the viruses of tobacco mosaic and Aucuba mosaic. It is significant that in all the known instances of this type of acquired resistance the individuals of the pairs of viruses have many properties in common. The first was recorded by Thung (1931). This worker showed that tobacco infected with the virus of common tobacco mosaic, if further inoculated with the virus of " white mosaic," failed to develop the symptoms of the latter. Both viruses are of the tobacco mosaic type. Salaman (1933) has found that tobacco and *Datura* infected with a weak strain of potato virus " X " give no further reaction when reinoculated with a virulent strain. These viruses are so similar that Salaman considers them to be biotypes of the same virus. More recently it has been shown by Kunkel (1934) that plants of *Nicotiana sylvestris* infected with the virus of tobacco mosaic fail to develop local lesions when inoculated with the Aucuba virus. Here, also, the two viruses are both of the tobacco mosaic type.

If viruses with similar properties do affect plants in the same manner it is only to be expected that viruses with widely different properties will affect plants differently, and it is of some interest to note the results obtained by infecting plants with such pairs of viruses. Tomato plants infected with potato virus " D " when further inoculated with the virus of tobacco mosaic develop an acute necrotic disease. Potato plants infected with potato virus " A " develop severe crinkle symptoms when inoculated with potato virus " X," and tobacco plants infected with virus

" X " develop a crippling necrotic disease when further inoculated with potato virus " Y." The individuals of all the pairs of viruses cited differ greatly from each other in most of their properties, and each not only fails to protect infected plants against further infection with the other, but the two acting together produce a far more severe disease than either does alone. If these viruses have different metabolisms and affect different materials in the plant it is obvious that the presence of one in no way affects the multiplication of the other, and, as the plant is then being attacked at two distinct sites, the disease resulting from the presence of both will be more severe than that caused by either acting alone.

It is believed that the acquired resistance must in some manner depend on the similar physiological requirements of the viruses, for no destructive action of one virus on the other has been obtained. When sap derived from tobacco infected with viruses " D " and " X " was mixed *in vitro*, allowed to stand overnight, and then inoculated to tobacco, both viruses entered the plants. The symptoms resulting from this infection were mixed, and consisted of both necrotic rings and chlorotic mottle, and both viruses persisted until the death of the plants. They did, however, tend to segregate, some areas showing symptoms more typical of one virus, and others areas of the other. By isolating these areas and inoculating them separately to healthy plants it was found possible to separate the viruses and to regain pure cultures of each. Thus although the two viruses can exist side by side in the same plant it appears to be impossible for them to be present in the same cell.

It has recently been suggested by Clinch and Loughnane (1933) that the viruses of the potato mosaic group might be divided into two groups, an " X " and " Y " group. If this should prove possible virus " D " would undoubtedly be placed in the " X " group. Its exact relation with virus " X," however, is difficult to determine. Some of its properties *in vitro*, *e.g.*, thermal death point and filterability, are the same as virus " X," but others such as dilution end-point and longevity differ widely. Out of a total of 28 potato varieties infected only three, Epicure, Arran Crest, and King Edward, give the same symptoms of disease as with virus " X." The reaction on tobacco is very similar to that caused by an extremely mild " X " virus, but on potatoes the diseases produced are more severe than those caused by the most virulent strains of the " X " virus. The difference seems too great to warrant calling it merely another strain of the " X " virus, and the relation would seem to correspond more to that existing between two species of the same genus, rather than to that between two varieties of the same species.

The writer has great pleasure in expressing his gratitude to Dr. R. N. Salaman for permission to use the materials forming the subject matter of this communication, for his many helpful suggestions and for access to much of his unpublished work.

Summary

An account is given of a hitherto undescribed potato virus, provisionally named potato virus "D." In certain potato varieties it induces a characteristic necrotic disease, Foliar necrosis, the symptom picture of which is described. The following are the reactions of 28 potato varieties to infection with virus "D":—

Foliar Necrosis—Arran Cairn, Arran Chief, Arran Comrade, Arran Pilot, Arran Victory, British Queen, Edzell Blue, Katahdin, Kerr's Pink, May Queen, Rhoderick Dhu, President, and Sharpe's Express.

Top-necrosis—Abundance, Arran Consul, Arran Crest, Duke of York, Epicure, King Edward, Majestic, and Up-to-Date.

Carriers—Arran Banner, Arran Scout, Champion, Di Vernon, and Eclipse. Great Scot and International Kidney may also act as carriers, but the reaction is not constant. The symptoms produced on tobacco, tomato, *Datura Stramonium* and *Nicotiana glutinosa* are also described. No insect vector is known.

An account is given of the purification of the virus and of certain of its properties *in vitro*; it is filterable through an L 5 candle; it is destroyed by heating for 10 minutes at 68° C it withstands ageing in expressed sap for 5 days at 25° C, and for 6 weeks at 1° C; it is inactivated by 60% ethyl alcohol, 4% phenol, and 4% formalin, all acting for 1 hour; its dilution end-point varies from 1 in 1000 to 1 in 5000, depending on the species and age of the host plant.

It is shown that plants infected with virus "D" acquire a resistance to further infection with potato virus "X," and *vice versa*. The extent of this resistance varies somewhat and depends on the species and on the rapidity of growth of the host plant. The possible reasons underlying this acquired resistance and the relation between viruses "X" and "D" are discussed.

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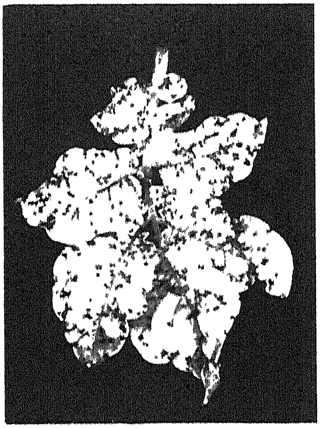

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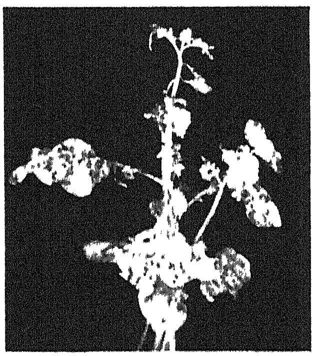

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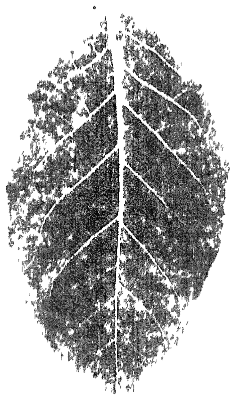

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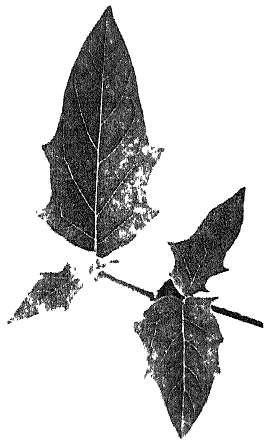

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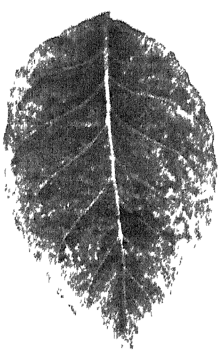

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Explanation of Plates

Plate 21

Fig. 1 Arran Victory leaf inoculated with virus "D," showing necrotic local lesions (infra-red plate).

Fig. 2 President plant showing first systemic symptoms of Foliar necrosis. The intermediate leaves are covered with necroses, which are most prominent on the undersurface (photographed by transmitted light).

Fig. 3—Arran Victory plant with Foliar necrosis, showing the middle leaves completely necrotic and wilting.

Fig. 4 President plant showing the "palm leaf" effect. All the middle leaves are necrotic and fallen, whilst the upper show scattered necroses and interveinal mottle.

Plate 22

Fig. 5—Arran Victory leaf in the second stage of Foliar necrosis, showing many scattered black necroses and deformity (infra-red plate).

Fig. 6—Top of Majestic plant infected with virus "D," showing commencing Top-necrosis (infra-red plate).

Fig. 7—Top of Up-to-Date plant infected with virus "D," showing severe necrotic stripes in stem and petioles (infra-red plate).

Fig. 8—Abundance plant in the second year of infection with virus "D." All the leaves are severely necrotic and are falling (infra-red plate).

Fig. 9—Tobacco leaf, var. White Burley, showing chlorotic mottle produced by infection with virus "D."

Fig. 10—*Datura Stramonium* infected with virus "D," showing faint interveinal chlorotic spotting.

Fig. 11 and Fig. 12—Five *Datura* plants infected with virus "D" and later re-inoculated with virus "X," and five control plants of the same age inoculated only with virus "X."

Fig. 13—Tobacco leaf of plant infected with virus "D" and later inoculated with virus "X" over the whole surface. In addition to the faint mottle many small necrotic rings (local lesions) are shown.

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The Effect of Salts on Cell Permeability as shown by Studies of Milk Secretion—(continued)

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(*Communicated by J. Mellanby, F.R.S.—Received September* 22, 1934)

It has been shown by Peskett (1933) that in a group of cows of the same breed certain of the differences in milk composition which are associated with different animals at the same time, or with the same animal at different times, can be correlated to some extent with variations in blood composition. In these experiments evidence of correlation between the ratio of solids-not-fat to fat in milk and that of sodium to diffusible calcium in blood was obtained. It was suggested, too, that the variations in milk composition may occur as a result of alterations in the relative permeability of the membranes of the mammary cells to lipoids and non-lipoids, caused by changes in the proportions of those salts in the blood. In keeping with the approximate constancy in milk composition that is generally observed among cows of the same breed, the variability of the blood and milk values was small in these experiments which were confined to cows of the Dairy Shorthorn breed.

With cows representative of a number of breeds, wider variations in milk composition are to be expected. In the following experiments we have extended our observations to include Ayrshire, British Friesian, and Guernsey cows in addition to Dairy Shorthorns. Of these, Guernseys and British Friesians occupy extreme positions as regards differences in milk composition, the former yielding milk very much richer in fat than the latter, while Ayrshires and Dairy Shorthorns are placed intermediately. The fat variations are associated with comparatively small differences in solids-not-fat content. In studying these different breeds we hoped, therefore, to provide some explanation of the marked differences in milk composition occurring between them, whilst confirming the earlier work under a wider range of conditions.

EXPERIMENTAL

While we have followed the general lines of the previous investigations, two important modifications have been introduced.

Firstly, we have tried to eliminate a source of error in the diffusible calcium determinations, namely, variable permeability of the collodion sacs used for dialysis, by adopting the following standardized method in making the sacs. For each batch of sacs a collodion solution was freshly prepared from a commercial preparation, known as "Necol." This was supplied in small containers which were opened only as they were required, the one consignment of containers being reserved for use throughout the series of experiments. To each 200 gm Necol were added 60 cc dry ether and 40 cc absolute alcohol. The resulting solution was poured into clean tubes which were covered with glass microscope slides until all bubbles had risen to the surface. The tubes were then drained with a rotatory movement for exactly 1 minute, allowed to dry for 30 minutes in an inverted position and finally for a further 30 minutes in the reversed (upright) position. Thereafter they were immersed in distilled water in the ice-chest, the water being renewed daily for the first three days. The drying process was always carried out in the same laboratory at a temperature of 60° F under similar climatic conditions, though the humidity of the atmosphere was not accurately controlled. After being used once the collodion solution was discarded.

Secondly, we have studied groups of carefully selected animals instead of individuals. This course was to some extent forced upon us by circumstances which limited the number of analyses that we could carry out simultaneously. It is obvious that the same result should be obtained from analysis of a composite mixed specimen prepared from the samples of individual animals as from the mean of the individual results from the same animals, though the former procedure unfortunately diminishes the variability.

The animals in each group were selected as follows. A number of healthy cows of one breed, free from udder troubles, nervous excitability, or marked irregularity in yield and composition of milk, were chosen and the fat and solids-not-fat percentages and yields of both morning and evening milk were determined in these cows for two or three consecutive days. On the last day, in addition, samples of jugular venous blood were collected from each cow as in the work reported previously, *i.e.*, about 2 hours after the morning milking. A group of animals was finally selected including only those which had shown reasonable regularity in their yield and composition of milk throughout the period of observation.

From the blood sera and the last evening milk samples of all the cows finally chosen for the group, composite mixed specimens of blood serum and of milk were prepared with extreme care, using an equal volume of

each of the individual samples. The determinations of fat and solids-not-fat in milk and of sodium, diffusible calcium and total calcium in blood serum were conducted in quadruplicate on these composite specimens by the methods used previously. The results are recorded in Table I and shown graphically in fig. 1.

Discussion

For simplicity we have omitted the ratios of sodium to non-diffusible calcium in blood from the data since they show no significant correlation with the solids-not-fat to fat ratios in milk, in agreement with the previous findings.

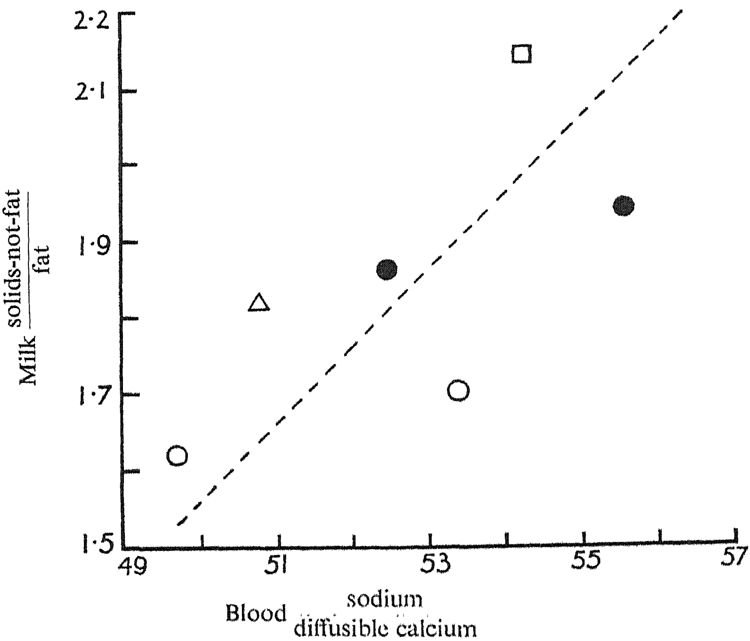

Fig. 1—Relation between solids-not-fat to fat ratio of milk and sodium to diffusible calcium ratio of blood in different breeds. ○ Guernsey; ● Dairy Shorthorn; △ Ayrshire; □ British Friesian.

Our results are specially interesting from the point of view of the marked differences in the proportions of fat and solids-not-fat which are found in the milk of different breeds of cow. The variations of these components that we have observed in the milk of the various breeds can be regarded as fairly representative of those breeds (*cf.* Drakeley and White, 1927), considering that our milk samples are from the afternoon milking

TABLE I—COMPOSITION OF COMPOSITE MIXED SPECIMENS OF BLOOD SERUM AND OF MILK

No. of group	Breed	No. of cows in group	Blood serum				Milk		
			Total Ca mg/100 cc	Sodium mg/100 cc	Diffusible Ca mg/100 cc	Sodium / diffusible Ca	Solids-not-fat gm/100 gm	Fat %	Solids-not-fat / fat
1	Dairy Shorthorn	12	10·5	297	5·66	52·5	8·35	4·49	1·86
2	Guernsey	6	10·7	309	5·78	53·4	8·89	5·23	1·70
3	British Friesian ..	11	11·0	306	5·64	54·3	7·93	3·71	2·14
4	Dairy Shorthorn	9	10·6	320	5·77	55·6	8·57	4·43	1·94
5	Guernsey	9	10·4	301	6·07	49·7	9·07	5·59	1·62
6	Ayrshire	12	10·2	292	5·76	50·8	8·58	4·72	1·82

and therefore comparatively richer in fat. The accompanying differences in blood composition are presumably equally representative of the different breeds, and since they are correlated with the differences in milk composition they probably play an important part in determining the character of the milk secreted.

Comparing the present results with those reported earlier (Peskett, 1933) the following points of interest emerge. The variability of blood and milk values of the different breeds studied in the groups is rather less than that observed previously in different cows of the same breed studied singly. It is unfortunate that we cannot continue these investigations and that, owing to the difficulties we have met in experimenting on cows other than those of the Institute herd (in which only Dairy Shorthorns and Guernsey are represented), the number of groups investigated has been fewer than we desired and limited to a narrower range of breeds than was originally planned. In spite of these limitations, however, the results as shown in fig. 1 exhibit a high degree of correlation between the blood and milk values, and it must be remembered that each point plotted is based on the analysis of material derived from a number of animals, which would doubtless have shown very wide variability had they been studied individually.

If, as shown in fig. 2, the data reported above are superimposed on the graph published previously for 15 Dairy Shorthorn cows studied on one day, the points added fit a straight line far more closely than did those obtained then, the two lines being almost parallel and not far removed from each other. This confirmation of the earlier results is the more striking when it is remembered that we are dealing with animals of four breeds, kept under different management on widely separated farms and that the variability of the blood and milk values is less than it was before. We attribute the satisfactory nature of our recent results largely to the improvements in technique that we have introduced.

As regards the intimate mechanisms involved in the regulation of the supply of milk precursors to the mammary cells, it was suggested previously that the membranes separating these cells from the blood could be likened to a twinkling mosaic, made up of two different types of emulsion (lipoid-in-protein and protein-in-lipoid), in a state of dynamic equilibrium and ever changing in pattern under various influences, such as the balance of antagonistic salts in the surrounding fluid. The new evidence presented substantially confirms the earlier results and is in complete agreement with the hypothesis suggested.

This hypothesis rests on the assumption that blood lipoids are the precursors of the lipoids of milk, an assumption for which further support

has been provided by the recent results of several independent workers, namely, Lintzel (1934), Blackwood (1934), and Graham and Kay (1934). Our findings being in agreement with our hypothesis are therefore consistent with the theory of the origin of milk fat from blood lipoid; we would hesitate, however, before quoting them as direct confirmation, as the gap between blood salts and the lipoids and non-lipoids of milk is very wide.

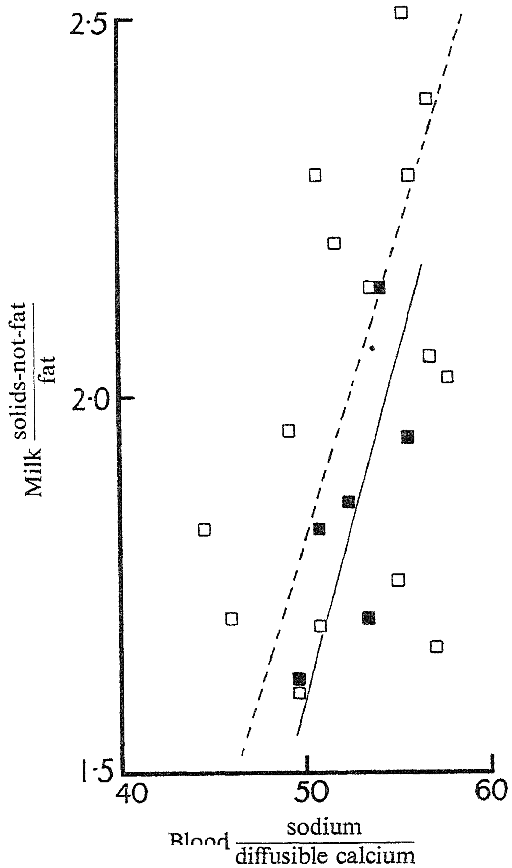

FIG. 2—Results obtained on four breeds in the present series of experiments superimposed upon previous results for individual animals of a single breed. □ Previous points for individual Dairy Shorthorn cows; ■ new points, as shown in fig. 1.

While much recent research has been directed towards elucidation of the nature of the milk precursors, the mechanisms governing their supply to the mammary cells have been overlooked. We wish to emphasize the importance of further investigation of the fundamental permeability

relationships involved, which are an essential link in the chain of events leading to the formation of milk from blood in the mammary gland.

We acknowledge gratefully our indebtedness to Mr. W. Craig, of Green Farm, Burghfield, and to Imperial Chemical Industries, Ltd., Jealott's Hill, for their assistance and facilities which enabled us to obtain samples from British Friesian and Ayrshire cows respectively.

Part of the cost of apparatus used in this work has been defrayed out of a grant to one of us (G. L. P.) from the Government Grant Committee of the Royal Society.

Summary

The correlation observed previously between the ratio of sodium to diffusible calcium in blood and that of solids-not-fat to fat in milk has been confirmed in groups of cows of four different breeds. It is probably an important factor in the causation of the marked differences in milk composition which occur among the various breeds.

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Address of the President, Sir Frederick Gowland Hopkins, at the Anniversary Meeting, November 30, 1934

Death has this year exacted a toll on our Fellowship which is heavier than usual. We have lost one distinguished Foreign Member and twenty-two Fellows. In the list are the names of two who did fine service for the Society on its Council and in office. Sir Arthur Schuster was a Secretary for seven years and Foreign Secretary for four. Sir William Hardy was Secretary during the difficult War years and after, serving from 1915–25. Each of these while in office gave loyal service to the internal affairs of the Society and by their enterprise made for it national and international contacts from which it gained in prestige and influence.

ARTHUR SCHUSTER, while a most able and successful investigator, displayed great capacity and foresight in the conduct of affairs. He foresaw the future which was so abundantly to justify the foundation of Manchester University as an independent institution, and he took a leading part in the movement which resulted in that foundation.

In 1888 on the death of Balfour Stewart he was appointed to the Langworthy Chair of Physics. His personal efforts greatly helped to make possible the erection of the fine physical laboratory in which he himself did much important work. More than this, it was largely due to Schuster that Manchester became the birth-place of nuclear physics. When he retired from the Chair in 1909 he saw to it that Rutherford should succeed him, and the great consequences of that appointment are familiar to all. Schuster's own scientific activities covered a wide ground. As a young man he was put in charge of the Solar Eclipse expedition that was sent out to Siam, and he came to share in the work of three other such expeditions. The main lines of his personal work, theoretical and practical, came under the heads of discharge through gases, spectroscopy and terrestrial magnetism, but his successes were not confined to these. Reference may be made for instance to his determination of the specific heat of water in terms of international electric units. He was always keenly interested in geophysics and meteorology, and when the Meteorological Office was to be reorganized, his advice was sought and welcomed. Schuster provided, personally, financial support to many scientific enterprises and, as we

cannot forget, was a generous benefactor to this Society. He was Secretary of the International Research Council for nine years, and a member of the Cambridge University Commission. He was elected Fellow of this Society in 1879, and was awarded the Copley, Royal and Rumford Medals.

The vital personality of WILLIAM BATE HARDY, the fertility of his mind, and the generosity of his ever ready help, stimulated the thought and activities of so many that he will be missed as few men can have been missed by their contemporaries. He began his scientific career as a student of zoology at Cambridge, and after graduation joined the staff of the progressive School of Physiology which Michael Foster had created. His special concern with teaching and research in histology and his realization that the current technique of this subject was open to criticism from the standpoint of colloidal science led to a deep interest in the latter and to researches which greatly advanced progress in certain of its more fundamental aspects. Later he turned to problems in molecular physics and studied the phenomena associated with static friction and afterwards those presented by lubrication and adhesion. On all aspects of the boundary state he became a recognized authority. In spite of his pre-occupation with physical phenomena he never lost his interest in the living organism, but took every opportunity of applying his knowledge of the former to problems presented by the latter.

During the earlier years of his duties as Secretary, the nation's food supply was in danger and it was his care that the Society should play its proper part in mitigating that danger. He kept it in touch with other organizations devoted to the same task, and the Royal Society Food (War) Committee formed through his initiative exercised an important influence on national policy. The experience of the various problems of food preservation and transport he gained at this time became widely recognized and resulted in his appointment as first Chairman of the Food Investigation Board and Director of Food Investigation in the Department of Scientific and Industrial Research. In all the activities of this important post his success was outstanding, gaining for him the confidence of administrators and industrialists in equal measure.

Hardy became a Fellow of Gonville and Caius College in 1892 and later he was for many years one of its Tutors. He was elected a Fellow of this Society in 1902 and in 1926 received a Royal Medal. At the time of his death he was President of the British Association.

William Hardy had many enthusiasms, but perhaps the greatest of all was that of the sailorman. He loved the sea in all its moods; but most he loved it when it was dangerous.

p. 405, line 10	*for* ‘ limits ’ *read* ‘ limbs ’
p. 405, line 11	*for* ‘ fœtid ’ *read* ‘ fœtal ’

JAMES COSSAR EWART was a pioneer in the study of hybridization and other problems of animal breeding. He held the Chair of Natural History in Edinburgh for forty-five years (1882-1927) and greatly developed there the Department in his charge. In 1882 he became scientific member of the Fishery Board for Scotland, and during the next seven years was the author or joint author of a large number of papers or reports dealing with fisheries problems. As a comparative anatomist he published important papers dealing with the electric organ of the skate, the cranial nerves and the lateral sense organs of this and other fishes. He later studied the development of the limits of the horse, and described that reduction of the digits during fœtid and early life which is of such great interest in view of the similar reduction which is known to occur during the evolution of the horse. About 1895 he began the work in animal breeding for which he became so well known. He devised careful experiments to throw light on certain of the problems of cross-breeding and in-breeding; on reversion and on telegony. Of particular interest were the results he obtained by crossing a Burchell's Zebra stallion with mares of various breeds of horses. Among many results of interest his experiments seemed to bring complete disproof of the existence of telegony. Ewart later published many papers on the origin and evolution of horses and ponies, and some of his most recent work involved investigations for the improvement of the fleece of sheep. During his latest years he was concerned also with the origin of feathers, and the relationship between feathers and scales. He was elected to the Fellowship of the Royal Society in 1893.

JOHN JOLY, Professor of Geology and Mineralogy in Dublin, was a man whose genius was displayed in a wide ground of intellectual activities. He was chemist, physicist, engineer, biologist, and contributed something of importance to all of these callings. He invented the steam calorimeter, and by its means contributed much to our knowledge of molecular physics, and his invention of the meldometer afforded valuable help to the science of metallurgy. Having worked out a method for extracting radium, he then applied his ingenuity in improving its use as a curative agent. It was Joly who made possible the deep seated application of radium by his suggestion for the use of hollow needles as containers. By consideration of the sodium content of oceans he made calculations as to the age of the earth, while his share in the study of the radio-activity of rocks and the thought he gave to the data obtained, has been claimed to be as fundamental in stratographical geology as the work of Darwin in biology. To the field of biology he also made contributions, for instance to the theory of the ascent of sap in plants.

All these activities and many more were those of a man who was deeply read in English literature, and was a keen Alpine climber and yachtsman. Joly had been a Fellow of the Royal Society for over forty years. He received the Royal Medal in 1910.

FINLAY LORIMER KITCHIN as palæontologist to the Geological Survey of Great Britain did much to advance both the scientific and practical sides of his subject. His wide knowledge and his critical acumen were of great value to the work of the Survey. He was almost unrivalled in his ability to interpret unpromising and even fragmentary fossils and his skill in this direction was strikingly displayed in connection with the borings made in the Weald during the exploration of the Kent coal field. The Geological Survey's memoirs published on this subject were probably the best known of Kitchin's official contributions. His advice and criticism were freely sought by his colleagues and by palæontologists in other circles. To all he gave ungrudgingly an immense amount of help. He was elected to our Fellowship in 1928.

DUKINFIELD HENRY SCOTT was a distinguished palæobotanist. Among other important work he collaborated with W. C. Williamson in producing an elaborate series of memoirs on the orientation of the fossil plants of the Coal Measures. He later published a series of papers on the structural affinities of fossil plants from the Palæozoic Rocks, and was a joint discoverer with Oliver of the existence in carboniferous times of seed-bearing ferns which form a connecting link between two great groups of the flowerless and the flowering plants. Scott's work was of the greatest service to all concerned with the study of evolution. He became a Fellow of the Royal Society in 1894, was Foreign Secretary from 1912 to 1916, and received a Royal and the Darwin Medal.

WILLIAM BARLOW was well known for his researches in crystallography. With Sir William Pope he carried out researches on crystal structure which did much to prepare the way for modern developments in which mensuration by means of X-rays has provided an experimental method of verifying the structures deduced from the theory of valency. Barlow also contributed various papers to English and German scientific journals on crystal structure, on the homogeneous partitioning of space, and on the connection between crystal structure and chemical composition. He was elected F.R.S. in 1908, and was a Fellow of the Geological and Chemical Societies.

DAVIDSON BLACK was honorary director of the Cenozoic Laboratory of Pekin, a post which he admirably served and in which he did high service to pre-history and anthropology. He held the position of Assistant Professor of Anatomy at Cleveland, Ohio, but determined to

come to England in order to study comparative neurology under Professor (now Sir Grafton) Elliot Smith. The Professor was at this time working on the reconstruction of the Piltdown skull and Black became profoundly interested in primitive man and his history. He had earlier been led to believe that China was a highly promising land for the discovery of man's earliest ancestors, and when in 1916 he was offered the position of Professor of Neurology in Pekin Medical College he saw the opportunity for enterprises that he had in mind, and immediately accepted the offer. In 1926 an expedition under Dr. Gunnor Anderson found an early Pleistocene tooth and Davidson Black was one of those who firmly believed it to be human. He published a detailed description of this tooth, and on the basis of the evidence it offered he founded a new genus and species. He proposed to call the species to which the tooth belonged *Sinanthropus pekinensis*. In 1928 excavations carried out by Dr. Berger Bohlin in conjunction with the Chinese geologists, C. C. Young and W. C. Pei, yielded two jaws in association with pieces of brain cases. The validity of Black's views as based upon the tooth thus became fully established. The treatment of these important remains was carried out by Black himself with great technical skill, and the photographs and casts prepared by him made it possible for other authorities to realize the nature of the discovery. He became a Fellow in 1932 and in the same year was Croonian Lecturer.

WALTER ROSENHAIN, whose death at the comparatively early age of 58 has robbed metallurgical science of one of its best-known leaders, was an Australian by birth. His reputation was made early when, as a student at Cambridge, he described, jointly with Sir Alfred Ewing, the mechanism of deformation of metals by slip. This was the beginning of a long series of researches in metallurgy, mainly carried out during his 25 years' tenure of the post of Superintendent of the Metallurgy Department of the National Physical Laboratory. As the head of a team of workers he did much to improve technical practice, especially in the light alloy industry, and to lay the foundations of an exact knowledge of alloys by the accurate determination of phase diagrams, using highly purified materials. His work on the mechanism of crystallization and of mechanical deformation of metals, over a wide range of temperature, is fundamental, and even where his conclusions have not received universal assent they have actively stimulated research, here and abroad. His experimental skill and ingenuity led him to devise a number of laboratory appliances and metallographic methods which have been widely adopted. Rosenhain had a remarkable gift of lucid exposition, and was unrivalled in debate on metallurgical questions. An excellent

linguist, he took an active part in the international organization of his science, and was well known to his foreign colleagues. He had great qualities of leadership, and his strong personality made a great impression on the committees and institutions to which he devoted much of his time. He was elected a Fellow in 1913.

FRANCIS ARTHUR BATHER. After nearly forty years of work in the Department of Geology at the British Museum, and after serving as Deputy Keeper for twenty, Bather became Keeper in 1924. As a palæontologist his special interest was centred in the fossil echinoderms and especially in the crinoids. On these, as on other subjects, he published papers notable for careful description and felicity of diction. As a Curator he was remarkable for his insistence upon meticulous care in matters of detail whenever he thought that even minutiæ were important. He was immensely interested in museum technique and fully versed in its developments at home and abroad. Another of his enthusiasms was for scientific journalism, and he always demanded that high standards should be maintained by those who pursue it. He himself at one time edited *The Museums Journal*, and was the author not only of carefully written technical contributions to it and other Journals, but also of delightful reivews published in *The Times Literary Supplement*. Bather was elected Fellow of the Royal Society in 1902.

Sir THOMAS MUIR, died in his ninetieth year at Rondebosch, in South Africa. After a highly successful career in Scotland as a mathematical teacher he became Superintendent General of Education in the Cape Colony in 1892, a post which he held until he retired in 1915. His administrative work, which was carried out with thoroughness, has left an abiding and beneficial mark upon South African education in all its branches. During his years of teaching and of later official duties he developed and cherished an interest in algebra, and more particularly in the history and development of determinants, a subject upon which he has for long been acclaimed as the greatest authority. Such a work, carried on for half a century and continued through his nineteen years of retirement almost until the eve of his death, has played no small part in furthering the study of higher algebra initiated last century by Cayley and Sylvester. Muir's five volumes on the history of determinants stand as a monument of devoted research, fruitful suggestion and penetrating criticism, such as have won world-wide admiration. It has been given to few men of science to write on a technical subject with such charm and literary skill. A natural accompaniment of such a work has been the acquisition of an extensive library. This, said to be one of the finest mathematical collections in the British Empire, has been bequeathed to the South African Public Library.

Muir was a scholarly musician, had a gentle kindly manner and a keen sense of humour. To the end he was courtly, sympathetic and charming, with a wide knowledge of men and of books, his sight and hearing well-nigh perfect, and upright as a pine in his plantation at Rondebosch. He was elected a Fellow in 1900.

With the death of SIDNEY HOWARD VINES at the age of eighty-four, the Society loses a link with the time of Huxley, under whom Vines was a demonstrator in 1874. After graduating at Cambridge he studied under Julius Sachs at Würzburg and on his return to England started a botanical laboratory at Cambridge. After five years as Reader in botany at Cambridge he was appointed Sherardian Professor of Botany at Oxford, and under his guidance for 31 years the importance of botany in the University curriculum steadily increased. While he was specially devoted to physiological work, he was also a master of morphology. He was made a Fellow in 1885.

ARCHIBALD BYRON MACALLUM may be regarded as the pioneer in Canada of teaching and research in General Physiology. In his researches he early turned his attention to the demonstration of the local distribution of various elements especially iron, phosphorus, potassium, calcium and chlorine in cells and tissues. For this purpose he developed various methods of micro-chemical technique. In particular he made a very thorough investigation, extending over several years, of the distribution of potassium in plant and animal cells, and he found that the element is concentrated in regions in such a manner as to suggest that surface tension plays a great part in determining this distribution. He was always inclined indeed to relate many of the activities of the cell to the influence of surface tension, and he was responsible for a theory of muscular contraction in which that factor played a predominant part. Numerous estimations by him of the percentage amounts of inorganic ions in the tissues and body fluids of various animals led him to remark that in respect of the relative concentrations of these ions there is a striking resemblance between the ocean and mammalian blood plasma. He advanced the view which has received much attention that this was an indication of the oceanic origin of land animals. Macallum was always interested in the broader aspects of biology, a circumstance that made his teaching very stimulating. His influence upon Canadian science was great. Among other important offices that he held was that of Administrative Chairman of the Advisory Council for Scientific and Industrial Research of Canada. He was elected to the Royal Society in 1906.

BERTRAM DILLON STEELE held posts in Melbourne, Montreal and Edinburgh, but will be especially remembered for his work as the first

holder of the Chair of Chemistry in the University of Queensland. During the war he returned to England and applied his chemical knowledge in the service of the Ministry of Munitions. Among his more important contributions to science are the researches on the electrochemistry of non-aqueous solutions and the design of a very sensitive microbalance in conjunction with Kerr Grant; while it may be that the general public will remember him chiefly in connection with the control of the prickly pear in Queensland.

ARTHUR PRINCE CHATTOCK, Emeritus Professor of Physics in the University of Bristol, was a man of such modesty and retirement that it was perhaps only those who were brought into personal contact with him that fully realized his marked originality, experimental ingenuity and powers of exposition as a teacher. The volume of his published work was relatively small, but this was due partly to the fact that during much of his life he rarely had more than one assistant, and partly to his extreme caution in going to print. But the majority of his papers, notably those on the discharge from points and on the gyromagnetic effect, will have a permanent place in the literature of physics as pioneering investigations. It was also the Chattock-Fry pressure gauge that first enabled an insight to be obtained into the way in which the pressure varies over the surfaces of aeroplane wings and stream-line bodies. A perusal of some early notebooks of unpublished work shows that he was always thinking on novel lines, and had circumstances then placed him in a large laboratory where these ideas would have become known to a number of co-workers, they would undoubtedly have influenced the thought of the day. During a period first of temporary and later of final retirement from University life, he also became interested in problems of chicken incubation, and in animal psychology and published some papers in this field. He was elected a Fellow of our Society in 1920.

LEONARD COCKAYNE, an accomplished botanist, had been engaged in the economic study of forests in the New Zealand State Forest Service since 1919. During his earlier years he was wholly occupied in teaching, but in 1885 took up work at a private experimental station and continued investigations there for twenty years. A recognized authority on all forestry questions, his knowledge made his advice of great value to the various Forest Commissions on which he sat. He was elected to our Fellowship in 1912, and received the Darwin Medal for his contributions to ecological botany.

MARCUS SEYMOUR PEMBREY was Professor of Physiology at Guy's Hospital, London, retiring from that post last year after a tenure of thirty-four years. After graduating in Medicine at Oxford, he worked with

J. S. Haldane for three years, and was then appointed Lecturer in Physiology in the Charing Cross Hospital Medical School, where he stayed until he went to Guy's four years later. The powerful influence of Haldane produced a permanent effect upon Pembrey's work and thought. He dealt almost always with the physiology of the intact living animal, believing that the most important lessons were to be learned from such studies. In his earlier work he dealt with the gradual acquirement by the developing animal of the power of regulating body temperature; comparing, for instance, the effect of external temperature upon the respiration of the chick before hatching with its effect at different intervals afterwards. He also compared the temperature responses of new born mammals with those of the same animals some days later. Perhaps his most important work was that upon the respiratory activity of hibernating animals. He determined their respiratory quotients during their sleep, during the process of waking and after it. Pembrey became fully convinced that the facts thus observed showed that during the winter sleep fat is converted into glycogen, whereas during preceding periods, when the animal is preparing for its long sleep, the converse change occurs. He did a great deal of work upon the respiratory activity of man in all sorts of circumstances, normal and pathological. Pembrey was elected a Fellow in 1922.

Aeronautical science has suffered a severe loss through the accidental death of HERMANN GLAUERT at the early age of 42 years. Glauert left Cambridge to join the Scientific Staff at the Royal Aeronautical Establishment, Farnborough, in 1916. He served continuously there for 18 years, during which time he added considerably to the theory of aerodynamics and fluid motion. Perhaps his most important contribution to science was his extension of the potential theory of fluid motion, and its application to practical problems. He developed the vortex theory of aerofoils, and put forward a theory of the autogiro which has won general acceptance. The whole of his work is characterized by a clear grasp of fundamental issues. He had a flair for finding approximate mathematical solutions giving the essential results, and several of his approximations stimulated mathematicians at home and abroad to find more formal solutions, and by doing so to confirm the accuracy of Glauert's deductions. Glauert was elected a Fellow of Trinity College, Cambridge, in 1920, and a Fellow of the Royal Society in 1931. At the time of his death he was Head of the Aerodynamics Department at Farnborough.

It would be an interesting subject for a psychologist to study as to how the creative faculty and the critical faculty can exist simultaneously

in the one individual. Certainly the late WILLIAM McFADDEN ORR possessed both in the highest degree. Senior Wrangler in 1888 and Professor of Pure and Applied Mathematics at the Royal College of Science and afterwards at University College, Dublin, until less than a year before his death at the age of 68 years, he led a life of unremitting labour in various branches of Mathematics. His original contributions to Bessel Functions, Fourier Analysis, Stability of certain Liquid Motions, etc., all contained results which seem destined to be of permanent value. As the years went on, his critical faculties became more pronounced and he was able to elucidate many difficulties and rectify many conceptions, even in the writings of distinguished mathematicians. These qualities made him of the highest value as a referee for learned bodies. Thus, although much of his life work was unseen by the general mathematical reading public, yet his loss will be keenly felt by many of the greatest names in his subject. He was elected a Fellow of the Society in 1909.

WILLIAM MITCHINSON HICKS has rendered distinguished service in the domains both of Physics and of university education. One of the Cambridge School of mathematical physicists, he began, while a Fellow of St. John's College, the mathematical researches on the theory of vortex rings which quickly brought him into eminence. By his investigations on the toroidal functions necessary for their treatment, and by his discoveries of the possibility of existence of hollow vortex rings and of vortex aggregates, he has greatly advanced our knowledge of vortex motion. During the latter half of his life, and right up to his death at the age of 83, Hicks applied himself steadfastly to extending the discoveries of empirical relations between the frequencies of spectral lines. His essay on the Analysis of Spectra gained the Adams Prize in 1921, and his formula for the frequencies of the lines of a series has been of great value in spectroscopic work. In 1883 Hicks was appointed Principal and Professor of Physics and Mathematics at the Firth College, Sheffield, and from that time onwards he devoted himself to the furtherance of university education in that city. To his foresight and unremitting work is due in large part the growth of this small institution first into a University College and in 1905 into the University of Sheffield. The high esteem in which he was held was marked by his election to be the first Vice-Chancellor. Before long however, he resigned this office to devote himself as Professor of Physics simply to the teaching and research work which all his life he had preferred. He was elected to our Fellowship in 1885, he served on the Council for three periods, and in 1912 was awarded the Royal Medal.

GEORGES DREYER was Professor of Pathology at Oxford until his death. He was educated in Copenhagen and began pathological work while serving as a Medical Officer in the Danish Navy, becoming an expert in the production of diphtheria anti-toxin. While Privat-Dozent in the University of Copenhagen he carried out researches in a very wide field. He was appointed to the Chair of Pathology at Oxford, of which he was the first holder, at the early age of thirty-four years, and he put much energy into the development of his department. During the War he spent much time in France, and developed there the technique which he had previously established for the accurate diagnosis of enteric fever. Dreyer was responsible for the replacement of the single anti-typhoid inoculation of the troops by the much more effective triple inoculation. Later he transferred to the air force, and designed an ingenious apparatus for automatically controlling the supply of oxygen to the pilots in accordance with changes in altitude. After he returned to Oxford he concerned himself more particularly with assessments of physical fitness by vital capacity measurement in correlation with certain body measurements. After 1926 much of his energy was spent in developing the new laboratory built by a generous grant of the Trustees of the late Sir William Dunn. He was elected a Fellow of the Royal Society in 1921.

TANNATT WILLIAM EDGEWORTH DAVID, Professor for thirty-three years in Geology and Physical Geography at the University of Sydney. Born in Cardiff and educated at Oxford he went to Australia in 1882 to join the New South Wales Geological Survey of which he later became Director. The discovery of the great coalfields at Maitland was largely due to him, and it was he who saved these very valuable deposits from being alienated from the Crown. As is well known he joined the Antarctic Expedition of Shackleton, and it was he who, with Sir Douglas Mawson and Dr. MacKay, reached the South Magnetic Pole on January 16, 1909. During the expedition David and R. E. Priestley, the other geologist with the expedition, obtained important knowledge of the geological structure of Antarctica. His services during the War were of much importance. He was appointed geologist to the British Armies on the Western Front, where his expert knowledge was of the greatest service in mining operations. Edgeworth David was a brilliant teacher and many of his pupils made important contributions to the progress of Australian geology. He was elected to our Fellowship in 1900, and in 1920 he received the honour of K.B.E.

The death of SANTIAGO RAMÓN Y CAJAL removes a figure of exceptional and picturesque originality. From a village childhood in the Pyrenees, with scanty schooling and thence on to impoverished University instruction

and opportunities he passed suddenly at middle-age to the forefront of scientific attention as a supreme analyst of the structure of the nervous system. Beyond the untoward circumstances which beset his discoveries in their making they had the further difficulty for recognition that they appeared in Spanish when Spanish was far less familiar to scientific circles than, owing in part to Cajal's own work, it is now. But his observations were too important to misfire long. They pulled down much but they built up much more. By the beginning of this century they had reshaped our knowledge of the cellular architecture of the brain. Those seemingly inextricable networks hitherto regarded as a continuum of diffuse and indeterminate conduction they resolved into pathways perfectly definite and determinate. He read with his microscope the course and direction of the main paths through the cortex of the brain and other great masses of the nerve-centres. This was a great analysis; but he achieved another not less difficult in that other cell-system, which like a beneficent parasite grows with and imbeds the brain itself and unfortunately is so frequently a source of tumours. Here he founded the modern knowledge of brain tumours. He was a great Spaniard. Apart from unwearied devotion to research Cajal's main preoccupation lay in advancing the cultural progress of Spain. In that his influence and example were immense. He was a strong man of strong feeling. To the very end of his long life he cherished with unabated kindliness the memory of the early recognition of his work by this country as evidenced by the invitation sent him by the Society to give the Croonian Lecture before it in 1894. He was elected a Foreign Member of the Society in 1909.

I will now call your attention to certain points in the Report of Council. You will be glad to remember that the Society afforded hospitality to two important organizations which met in London during the autumn—the International Union of Scientific Radio and the International Union of Physics, the meeting of the latter being associated with a Conference organized by the Physical Society. There is general agreement that each of these gatherings was exceptionally successful. A matter for satisfaction is the circumstance that the Government generously extended its hospitality to both. It is, I think, most important that Government Departments should fully realize the value which accrues from such conferences when held in this country. They inform foreign visitors concerning its scientific equipment and activities which, as abundant evidence shows, are, to our detriment, often undervalued abroad.

The Report comments on the circumstance that the Annual Statement

of Accounts is presented this year in a form differing from the customary. Having now heard that Statement you will realize that the Society is greatly in debt to its Treasurer for the labour he has spent upon it. Our many Funds are now so logically classified as to bring clarity into their complexity. Probably not every Fellow of the Society has hitherto appreciated the magnitude of our expenditure on research, which has been somewhat obscured in earlier financial statements. The capital value of our Research Funds is to-day well over £600,000 and the income they yield is £23,700. A little over £25,000 has been actually expended on the support of research during the year; this slight excess of expenditure over income being fully justified by circumstances. These large figures are surely impressive and afford reason for satisfaction. A novel feature of Council's own Report this year is to be found in the personal progress-reports it contains from the various investigators who have received during the year allotments of over £100 from our Funds. These make clear the very wide and fertile fields of enquiry which it is the Society's privilege to support. It is proposed, I trust with your approval, that the publication of this information shall continue as a feature of Council's Annual Reports.

Although our research income is satisfactorily large, I am bound to add that it is far from being in excess of the claims made upon it. In particular it has proved very difficult this year to meet many requests for financial support for Expeditions of various kinds. Field Research is becoming an urgent need in many progressive branches of science and is, of course, costly. The Treasury when consulted on this matter some time ago did not see its way to increase the Government Grant by a sum ear-marked for this particular purpose, yet the existing amount of that Grant affords little scope for the support of Expeditions. This year some of our own Research Funds have therefore been raided (doubtless legitimately) for that purpose. It should, I think, be publicly known that Field Research of great scientific and economic importance is inadequately supported at the moment. As the Royal Society has supported Expeditions ever since it supported those of Captain Cook, a century and a half ago, it may claim to be well qualified to act as Trustee of any Funds that may be provided.

You will note that among donations for special purposes received by the Society during the year there is one from our Fellow Sir Robert Hadfield for the benefit of the Scientific Relief Fund, a Fund most worthy of support.

It has become customary that in an Annual Address from your President there should be some adequate reference to recent scientific

progress. I trust I may have your approval if on this occasion with a certain intention in mind I depart from that custom and deal chiefly with a chapter of history, together with some considerations which while primarily of importance to Medicine, are not I feel outside the concern of the Royal Society.

Nevertheless with the remarkable discussions at recent conferences still in mind it is scarcely possible to proceed without a passing reference to the extraordinary progress which atomic phsyics continues to make. The past year cannot fail to be memorable for the advances it has seen, yet so exciting is the position at the moment that it may well be followed by years fated to be still more memorable. Such are the complexities in atomic structure now being revealed, and so remarkable are the responses of atoms to certain forms of treatment, that one wonders with what objective picture of an atom (if any) the next generation will start its thought and work.

To progress on the biological side I will also make but brief reference. Out of the many instances of recent advances that might be chosen there are two which, taken together, will in a sense serve as a text for the rest of my Address.

Our Foulerton Professor, Dr. Adrian, with his colleague Mr. Brian Matthews, by means of the oscillograph method which the latter has done so much to develop, has been analysing the electrical changes which take place in the brain. Professor Adrian's special aim has been to relate the potential changes which can be recorded from the brain's surface with the changes in individual nerve cells. In this success has been attained. Adrian and Matthews have been led to repeat and extend the observations of a continental observer, Dr. Berger, on these potential changes as they are recorded from the heads of living subjects. It is fascinating to see these rhythmic activities of a human brain recorded as a series of waves in a graphic record after passing through the skull.

Adrian and Matthews have found that certain of these waves as observed by Berger arise in the occipital lobes and are due to the rhythmic activity of some part of the visual mechanism. It is noteworthy that concentrated thought, such as is involved, say, in mental arithmetic, temporarily abolishes the recorded rhythms which would thus seem to be characteristic of the quiescent organ. It is suggested, however, that this may be due to the circumstance that for the changes to be strong enough for registration with the apparatus in use the cells of the cortex must be beating in unison. Effort may localize them. Research on these remarkable lines is as yet new however, and striking developments may be expected. It is not impossible that when further developed the

technique employed may serve the clinical investigator concerned with the brain, even as the cardiograph has served those concerned with the heart.

As a second illustration I will refer to progress in the study of the virus of influenza. Many may remember that after previous very successful dealings with dog distemper Laidlaw, our Royal Medallist of last year, has with Andrewes, and Wilson Smith, working at the National Institute for Medical Research, applied similar methods of study to human influenza. A year ago they found that the disease could be transmitted to the ferret, and, profiting by this discovery, they demonstrated conclusively that the prime cause of this disease belonged to the group of filtrable viruses. As is usually the case when a human disease can be transmitted to an experimental animal the study of influenza has now become progressive. It was soon shown for instance that antisera could be prepared, capable of depriving the virus of its infectivity. These authors have now shown that the mouse can also be infected and the availability of this cheap and easily handled animal has at once made the approach to various aspects of the problem much easier.

The final issue of this now progressive research on influenza cannot, I think, fail to be of prime importance alike to Clinical and to Preventive Medicine. It illustrates afresh the nature of the help that the laboratory, and in such cases, the laboratory alone, can afford to the progress of medical knowledge. As the investigation was, of course, inspired by clinical experience it also illustrates the interdependence of the ward and the laboratory which has now received very general recognition.

It is a circumstance familiar to most that practice in the ward and activity in the laboratory, which but a generation ago made few contacts, have now come into close relations, with a degree of mutual respect between those who pursue one or the other that was perhaps lacking in the past.

It seems, however, that the minds of some physicians have been disturbed by one aspect of this new orientation. Not doubting that the laboratory has assisted and must continue to assist the growth of medical knowledge, they have felt that the introduction of multitudinous laboratory methods into the domain of diagnosis is tending to destroy the true Clinical Art, the art which for success in practice is only second in importance (if it be second) to clinical knowledge. It has been said that "the older physicians with minds undisturbed by a crowd of scientific facts developed a clinical sense, *sui generis*, as subtle as the sense of taste or the sense of smell, and it was upon this sense (I quote from an article once published

by *The Times*) that the great school of English Medicine was founded."

I do not know whether there is truth in the view that reliance on laboratory reports tends to destroy this sense. I have read, however, that even when Laennec first introduced the stethoscope there were some who complained that its use was fatal to the dignity of the physician and brought only discomfort to the patient. Each new diagnostic aid from the laboratory, in the opinion of some few, might well come into the same category. One would think, however, that the clinician possessed of that clinical sense should be able to use every effective aid in diagnosis and yet employ his special gift. It is his task and his alone to make a final and often difficult synthesis from various lines of evidence.

I am not qualified, nor do I propose, to say more upon this matter of laboratory aids to diagnosis, but I have been tempted to devote a major part of this Address to a consideration of another side of the relations between ward and laboratory, namely, the respective shares which they are likely to take in the future development of fundamental medical knowledge. That advances in such knowledge of first-class importance have followed upon advances in pure science pursued for its own sake is a claim which brooks no denial; it is a familiar theme.

At the same time we must recognize that there are some who see danger to-day in relying over much on the laboratory as the source and field of progress in medical science. Great emphasis is being placed just now on the circumstance that distinct from all the laboratory science which is ancillary to Medicine there is Clinical Science, *sui generis*, the progress of which depends on the direct and intimate study of disease as manifested by human beings. This, of course, must be the concern of clinicians and of clinicians alone, and it is felt by not a few of their number that proper recognition of its importance has failed of late. They urge in face of the rapid and almost intrusive progress of laboratory research that Clinical Science as defined must enjoy that independent status which is allowed to other branches of scientific activity. Its adequate pursuit calls for clinicians protected from the claims of practice but provided with wards or clinics. There they must practise something of the Clinical Art and yet function as trained and skilled investigators. Investigators so qualified and so equipped, while fortunately not unknown in this country, are rare, and the current re-awakening to the importance of what we may agree to call Clinical Science is stimulating a policy which aims at training them in greater numbers and providing them with proper equipment. The need has been expressed for the endowment of "a phalanx of trained clinicians who shall bring clinical science to a new pitch of scientific

efficiency and hold it there." If this is to become a national policy on the scale which seems to be suggested it must be recognized as one calling for quite exceptionally large endowments. Rightly or wrongly therefore, the pursuit of that policy may transfer to the ward or clinic much of the financial support which might otherwise be enjoyed by research in the pure sciences ancillary to Medicine. If this is only a possibility, it is one of which the consequences should be so far as possible foreseen. Full sympathy with the aims of the policy in question, ideally viewed, may yet go with some fear of the issue, and especially of a distant issue if it be developed too hastily or too far at a time when national resources are restricted.

I feel that questions which may thus arise are not without interest for the Royal Society which has been entrusted with large bequests for the express purpose of supporting original research in medicine, devoted to improvement in the treatment of disease and the relief of human suffering. The greater part, though not the whole of these resources, is being devoted by the Society to the support of fundamental researches and not to those *ad hoc* investigations which must be the business of Clinical Science as understood. Is that policy, it may be asked, open to criticism? The Society may in any case take pleasure in knowing that two of its own Fellows having enjoyed the freedom and the equipment necessary for the pursuit of Clinical Science have proved by their brilliant contributions to knowledge that within that specialized discipline work of the greatest importance may be done. Sir Thomas Lewis and Dr. Edward Mellanby will, I am sure, excuse this personal reference.

The successes of Clinical Science in such hands and in certain fields are indeed notable. Yet is it not true that the fields open to its methods of investigation, methods, that is to say, which are applicable to the living human body, must always be very limited when compared with the whole field of knowledge to be cultivated in the interests of Medicine? In a sincere desire to know how the future of this specialized branch of science is viewed by those who wish for a large increase in its personnel I have turned to the writings of those who do so wish, and who have doubtless pictured for themselves the field of its future activities.

I find that Sir Thomas Lewis has divided these activities into three categories. The first is the "Discovery of Disease" which he defined as a pursuit standing for the clear description of specific diseases or states so that these may be identified by others. The second category comprises experimental work on clinical cases; and the third the application of physiological discoveries to human material. This last category

calls for no comment here. In discussing the first I will ask you to follow the fate during the history of modern Medicine of a particular intellectual standpoint. The discovery of disease in the sense defined has been the aim of enlightened clinicians ever since Medicine made in the seventeenth century its escape from Galenic authority. But in so far as the definition implies the existence of diseases as independent entities it is of profound and, I think, of quite general interest to note how the reality of such a conception has been supported, challenged and reasserted throughout the history of modern Medicine. Does the identification of any disease as something specific mean that it is a real entity or is it at most a convenient abstraction? This question has provided ground for vehement controversy between schools of thought differing in fundamental outlook or affected by the influence of varying milieux. The history of this conflict can be plainly followed in an admirable book, the "Nosology" of the Danish author, Knud Faber.

How ill-equipped was the medical mind regarding the nature of disease at the time when the experimental study of nature began and this Society had its birth is illustrated by the terrible professional treatment suffered by our Royal Founder as he lay dying. Arthur Bryant's apparently well-documented account of the scene induces nothing short of horror. One wonders intensely how William Harvey would have viewed that scene. The Galenic teaching, still influencing the practice of his contemporaries, was, we know, contemptible to him. It is hard not to believe that had Harvey lived for five years longer his name would have been among those of our Original Fellows. His intimate friend, Dr. Ent, was of their number.

Harvey was a clinician but he was also instinctively a believer in the virtues of experiment and, as we know, supported his great theme by endless observations and experiments upon a great number and variety of animals. It has nevertheless been claimed that he was the father of "Clinical Science" and the claim need not be disputed.

The list of our original Fellows who joined the Society in 1663 comprises 146 names, of whom 24, or a sixth of the whole, were those of practising physicians. Ent, the friend of Harvey, and James Goddard, noted for the possession of a laboratory of which the Society made use, were on the first Council. Among others of great contemporary eminence were Francis Glisson and William Croone.

But there was one truly great physician who though a close friend of Robert Boyle, and doubtless very familiar with the advent of the Society, failed to join it though he lived for a quarter of a century after its foundation. This was Thomas Sydenham. Even a little familiarity with

Sydenham's writings will explain his abstention. He was a man who not only rejected the authority of the past but who hated theory and any kind of deductive speculation. For those who ignored the good work of Harvey the only kind of science which at that time had intruded into the field of Medicine was the pseudo-science of the iatro-chemists, and for that Sydenham had rightly no use whatever. He probably did not realize that the group of experimentalists then for the first time assembling were to initiate the growth of a kind of science very different from that which he mistrusted. With all his merits he was not, I think, possessed of great vision. But what we have to recognize is that Sydenham was a pioneer in that discovery of disease of which we are thinking. He loved system and, believing that each disease is an entity quite apart from the particular patient who displays it, he taught that the first task of the clinician was to reduce diseases to certain definite species with the same care that botanists were then using in their description and classification of plants. This was his own endeavour and he was the first to bring groups of related symptoms together and view them as having behind them an objective entity; something displayed by, but independent of, the patient—a specific disease. But Sydenham—if we except some of his views about treatment—was ever sane, and essentially scientific. Some of his lesser followers, however, pushed to absurdity the making and classification of species in disease. It was at one time taught, for instance, that there were twenty species within the genus Phthisis.

There was no outstanding advance in the theory or practice of Medicine during the 18th century though much was written concerning both. "What a vast literature," wrote Sir William Osler, "exists between Sydenham and Broussais. What a desolate sea of theory and speculation."

Real medical progress had indeed to wait till the rise of the great French school in the early years of the last century. At first most of the eminent physicians at Paris shared Sydenham's belief in specific diseases as entities, and classified them accordingly; but they added something to mere observation of symptoms as a basis for their classification. Bichat, for instance, one of the leaders of the school, was a profound student of pathological anatomy. He relied, moreover, on the microscope, the use of which may be said to represent the laboratory research of that day. Bichat emphasized the importance of relating the specificity of each disease to the nature of the fundamental tissues attacked rather than to disturbances as displayed in individual organs. But the Paris school as a whole adhered to ontological views. Each disease was for them an entity of which the pathognomic symptoms were to be carefully sought

and defined at the bedside. It was thus logical that this school should believe with Sydenham in the search for specific remedies to which they gave much attention. As we are thinking of one aspect only of medical history I must pass over the services of two great Frenchmen of this time, Laennec and Louis; those of the former to the art of diagnosis, and of the latter to medical statistics. But in this Paris school there arose one whose thought and teaching were rebellious. This was Broussais, mentioned by Osler in the remarks just quoted. Broussais vehemently protested against the conception of diseases as actual entities. They are, he urged, at best but factitious ones; metaphysical abstractions; no two cases displaying certain morbid symptoms in common are ever really the same. He held that ontological views inhibited progress and led to false treatment. Broussais' teaching had merit in its insistence that disorders of function should receive more attention amid the pathologico-anatomical studies of the day. He does not seem, however, to have influenced greatly the thought of his compeers in France, but his rejection of ontological conceptions was supported in important schools elsewhere.

Such was the contemporary school at Vienna which concerned itself not at all with the discovery of disease in the sense of which we are thinking. It ignored the demand of Sydenham and the Paris school that the clinician should seek to discover in each patient a specific disease and label it. His task, it taught, was rather to identify in the patient the various individual lesions which pathological anatomy had discovered or was discovering. A leading member of the school, Rokitansky, writing in 1846 claimed "that pathological anatomy should be the base not only of the knowledge of physicians, but also of their practice as it contains all there is in medicine of positive knowledge and the foundations of it."

Let it be remembered, however, that great English-speaking physicians, though certainly not protected from the claims of practice, were meanwhile busy at the bedside in the discovery of disease and brought about very real advances in clinical knowledge. In Ireland, Graves, Stokes, Cheyne and Adams; in England, Addison, Bright and Hodgkin, the physicians of Guy's Hospital. All these great men clearly demarcated the diseases and clinical conditions which are now indelibly associated with their names, and their identification of these as specific states has retained its full value to this day.

There remains for far too brief consideration the great movement in German medical thought which culminated in the middle of the last century. It was associated with a strong reaction against all personification of disease, and against all claims for specificity based on the mere

assembling of associated symptoms. The leaders of this movement taught that Medicine should employ the methods of physiology, for pathology is no more than the physiology of the diseased organism, while experiment is the ultimate and highest resource in pathological physiology. Virchow, whose powerful influence long kept these traditions alive, held that French and British ontology had stood in the way of real progress in scientific medicine, and believed that with its destruction the pernicious use of a therapy falsely called specific would also disappear.

It is only to this particular aspect of the German medical thought of that day that I can refer. It was a stage in the remarkable history, which I am asking you to follow, of a mental attitude.

The German school of thought in question stood, of course, for much more than this rejection of specificity in disease. Whatever its faults from the clinical standpoint, and some I know feel that its faults are too nearly being reproduced to-day, its scientific preoccupations certainly prepared the way for that truly progressive later period when the Viennese and German clinics attracted students from all over the world. It has been said with reason that the movement during the time of its dominance did harm to true bedside practice. If to look upon diseases as entities distinct from the bodies diseased was not philosophically justified at a time when no specific causal agent was known for any one of them, it is yet sure that the conception of individual maladies, however, abstract was essential in bedside practice if only for clear description and guidance in treatment. It is worthy of remark here that the logical French mind of Trousseau, a great bedside observer, was yet insisting, in 1861, when the so-called physiological school of Germany was exerting a great influence, that "the natural history of diseases resembles that of animals and plants; it deals in the same way with specific properties which separate the species." Trousseau thus went right back to Sydenham.

Almost immediately after this there came from the laboratory, whence alone it could come, knowledge which was to go far towards justifying Sydenham and Trousseau. The work of Pasteur did more to clarify medical thought in a vast field than most of the emanations from the clinical schools we have been considering.

I must not, of course, stop to discuss the growth and influence of bacteriology and micro-biology in general. The influence they have exerted is patent enough even to those who have no more than a superficial acquaintance with the progress of Medicine, clinical and preventive. In parenthesis it should perhaps be noted that the field of disease in which infection intrudes as a causitive factor, though wide enough, is somewhat narrower than earlier enthusiasm led us to believe.

So far, however, as my particular theme is concerned we have only to admit at this stage that a disease can justifiably be classed as a species when once its symptomatic display can be traced to a recognizable specific cause. Before Pasteur the only representative of such a cause was a mysterious something—something as it were divine—which Hippocrates himself had declared to be unknowable. Sydenham relied upon this, and so, explicitly, or more often implicitly, did the ontological thinkers who followed him. Pasteur replaced it in the case of infective disease by the highly objective micro-organism.

But we come at last to what would seem to be the final stage in this long history of what, following Sir Thomas Lewis, I have called the discovery of disease. Discussions concerning causation have always been troublous to the human intellect, and modern science tends more and more to elude them. In the clinical field there began some years ago a new emphasis on the constitutional factor in disease. A specific disease for instance follows upon the invasion of the body by a specific micro-organism; but not always. The presence of bacillus or virus is the efficient but not necessarily a sufficient cause of that disease. It is a familiar circumstance that we meet with carriers of an infective agent; folk who fortunately for themselves are so constituted that they harbour it without result, but unfortunately for others can transmit it. We may say then if we like, that constitutional lack of resistance is, no less than the presence of the infective agent, a link in the chain of causes. The constitutional factor is equally intrusive in the case of diseases of which the efficient cause is something other than infection. But the "constitution" of an individual depends, of course, upon many factors, inherited and acquired, and involves the relative functional efficiency of every organ in the body. It is doubtless susceptible of analysis by modern methods, and need not have for us that vagueness which attached to the old conception of a diathesis. It is complex in nature and yet must be in the thought of all who are concerned with the appraisement and treatment of diseases.

I have felt indeed that my justification for asking you to listen to so long a discussion is that this history of an age-long controversy may help to a realization of the greatness of the task of an individual to-day, if he is to advance knowledge as a clinical observer and also become an experimental investigator. In his approach to clinical appraisements he has to consider, not obvious symptoms alone, but the patient as a whole; his hereditary background, his environmental history, his psychological type. He has to remember that, as Broussa said, no two cases are ever the same. In his difficult synthesis the physician

of to-day has to remember the significance of data infinitely more numerous than those known to the old physicians whose praises were sung in my quotation from *The Times*. It would seem that he must possess or acquire in high degree that subtle clinical sense that was claimed for them, though with a mind no longer "undisturbed by a crowd of scientific facts." In acquiring the special quality of mind which this difficult synththetic power demands the clinical observer must be no specialist, and I cannot help feeling that the very structure of his mind must come to contrast with that of the successful experimental investigator whose thought must be analytical. Only exceptional individuals can, I think, excel in the double role.

Returning, however, to the categories pictured for the future activities of Clinical Science viewed as an independent pursuit, you will remember that the second category comprised "experiments on clinical cases." We all know that the method of controlled experiment wherever it can be applied is a shorter route to knowledge than the slow path of recorded observation. So far as it can be applied at the bedside to pursue it there to-day is one of the most praiseworthy of scientific aims. It would seem that in its application clinical science is most likely to attain to success denied to ordinary clinical observation, however enlightened. Let it be said in parenthesis that there is no thought of experiments which can do harm to patients. My own feeling, however, which may, I admit, be based upon lack of knowledge or of vision is that the fields in which really controlled experiments can be carried out on the intact human body are limited. Is there not indeed the same limitation of the field of controlled experiment when it is applied to the healthy body whether of man or animal? There is a great school of physiology which, in the belief that ultimate significance may be lacking from data obtained when any organism is studied in its parts, has largely confined its researches to a study of the living (and preferably human) body. That school has provided us with knowledge which could have been obtained by no other methods than its own. Its distinguished leader indeed has himself, without departure from his ideals, rendered supreme service to physiological science, and, by applying the knowledge he has won, equal service to the health of industrial communities. In awarding its Copley Medal this year to Dr. John Scott Haldane this Society has honoured itself in its recognition of so great accomplishment.

Dr. Haldane has shown what invaluable knowledge can be won from the living body by genius and skill when applied in well-chosen fields. It is nevertheless true that anyone who has followed the progress of physiology during the last twenty years knows that it has developed in

wide fields where experiments on the intact body of man could have had no possible application.

I am not able to assert that the field of clinical cardiology and related subjects which the work of Sir Thomas Lewis has illuminated is wholly exceptional in the opportunities it affords for experiment. I know that Dr. Edward Mellanby's studies of nutrition in relation to disease though based so largely on his field laboratory experiments have gained in importance by his work in the clinic while his results have a great future in their clinical applications. They offer a real illustration of that interaction of clinical and experimental work which is the theme of the admirable book he has written. There may be other experimental fields for Clinical Science of equal promise. I do not know, but I am voicing a suspicion that they are relatively few.

But I do know that there are many very wide fields in laboratory science the cultivation of which will continue to benefit Medicine and it is sure that the pursuit of pure science may at any time contribute to unexpected progress in entirely new directions. I will here display a fault in advocacy by choosing, for illustration, a field in which I am personally interested. I believe profoundly in the ultimate importance to Medicine of certain current activities in the laboratory which are relatively new.

In the history of all science which has dealt with living organisms a natural sequence may be traced. There is first the purely descriptive phase with the morphological studies which ultimately tempt efforts of classification. Then comes the study of function and the endeavour to correlate function with structure. Later the nature of the materials which support structure and form have received attention, and, later still, the endeavour has been made to follow the dynamic molecular events which underlie all displays of active function. Modern biophysics and biochemistry are busy upon the last task which, though not long begun, is to-day progressive and its progress is accelerating.

I am convinced that ultimately we shall attain to an adequate intellectual picture of these invisible events and of their organization in living tissues. Our thoughts will then penetrate below the surface of visualized phenomena. Disease itself will be viewed from a new standpoint. I believe indeed that even now those who think in terms of molecular events may have visions of progress denied to those whose thought is guided by the visible alone.

To the advancement of such knowledge studies of the intact body can contribute at most but very little. You will, of course, understand that I have throughout been speaking of the advancement and not the application of knowledge.

I will pause here to ask you not to look upon me as a mere obstructionist; I do not wish to see obstacles put in the way of activities in a field which, from the very nature of things, must always be of much importance. I would personally like to see a Chair of Experimental Medicine in every University capable of providing for such a Chair an adequate Clinic. More—if clinical science is to be encouraged without any discouragement of laboratory science, I would like the encouragement to be as generous as possible. I am only urging that in any planning for the future endowment of medical research proper consideration should be given to the relative magnitude of the fields in which new knowledge should be sought.

I seem to have sensed, however, the beginnings of a definite movement in this country, and indeed elsewhere, not, of course, to ignore the laboratory; but in the distribution of funds provided for medical research to endow the Clinic on a scale which might endanger the future of research in fundamental biological science. The tenor of my remarks has been due to a conviction that in the long run such a policy would sterilize advance.

I am tempted here to a quotation which I owe to Knud Faber. It is from the writings of the great French physician, Charcot. Charcot taught that clinical observation must ever remain the supreme court of jurisdiction on the clinic itself, but he says of it that "without scientific renovation it soon becomes a belated routine and, as it were, stereotyped." It was plain to Charcot, says Faber, that the fundamental sciences were the source from which clinical observation and clinical analysis must always derive their impulse for advance.

Encouragement of fundamental science is the special duty of the Royal Society. The Society is, nevertheless, entrusted with Funds with instructions to employ them for the advancement of medical knowledge leading to the relief of human suffering. It is well to recognize frankly that the laity, when anxious to support research directed to this end, is seldom aware of the influence of pure science on the progress of Medicine; a phrase in a will may therefore suggest limitations for the use of a bequest that the Testator with fuller knowledge would not have desired.

I will close by expressing the hope that the Society, when acting as Trustee in such cases will maintain a belief in its freedom to endow whatever field of research may at any moment seem to offer most help towards the progress desired, whether it be in the relatively narrow region of clinical science or in the wider regions of pure science.

Statement of Awards of Medals, 1934

Professor JOHN SCOTT HALDANE is awarded the COPLEY MEDAL for discoveries in physiology and their application to a number of important problems, practical and industrial, in which the human factor is involved. Haldane's researches in physiology centre on that of respiration. His work on the chemical regulation of breathing first made clear the delicacy of correlation on which depends the power of an animal to adapt itself so widely to environment, or to bodily activity. While this work has greatly affected the outlook of physiologists, and has had a fundamental influence on medicine, it has led also to applications of the greatest value, not only in science and medicine but in everyday life. These applications have shown no less skill and judgment, and have been no less imbued with the philosophical spirit, than his researches in pure physiology: indeed, they have continually acted and reacted with the latter. Thus his investigation of the causes of death in colliery explosions led him to his researches on the union of hæmoglobin with oxygen and carbon monoxide, thence to his discovery of the action of light on the equilibrium between these substances, a discovery which has played a fundamental role in the investigation of others. As another example, his interest in the influence of high underground temperatures on the working capacity of miners led him to an exhaustive study of the regulation of bodily temperature and of the function of the sweat glands. Haldane's contributions to problems of mining, ventilation and hygiene, and to measures to increase the safety of mines, have won him the gratitude and admiration of miners and mining engineers throughout the world. His "stage decompression," based on a bold and simple application of gas-laws to the human body, gives him a similar claim to the gratitude of divers, or of workers in compressed air, who are liable to caisson disease. The most striking characteristic of Haldane's work is the way in which his great experimental skill, based always on the simplest methods, and his strong philosophical instincts, have reacted with the broad humanity of his outlook and with the courageous use of his own person, when necessary, for the more drastic observations. The skill and the philosophical outlook have led to results of great scientific distinction: the humanity and the courage to results of great practical importance. Haldane's researches have influenced such diverse activities as mining, diving, flying, muscular work, mountaineering, protection against gas; and his teaching has left a permanent mark on physiological thought.

The RUMFORD MEDAL is awarded to Professor WANDER JOHANNES DE HAAS.

One of the main pieces of work that brought fame to de Haas was on the Einstein–de Haas effect, the measurement of which constitutes the first experimental proof of the spin of the electron. In association with a series of collaborators he has made an extended study of the magnetic susceptibilities of various diamagnetic solids in the single-crystalline as well as in the poly-crystalline form. These studies revealed a dependence of the magnetic susceptibilities on the strength of the magnetic fields used and also on the temperatures at which the measurements were made. Similar studies were made by de Haas of the electrical resistance changes undergone by diamagnetic substances under the influences of magnetic fields of various strengths. High diamagnetism and strong resistance-changes were found by him to go together. Von Laue, who had experimented for a long time on the refraction of light through a number of small holes, came to the conclusion that his results could only be explained by the use of the Quantum Theory of light. De Haas in 1917 showed that the phenomena could be explained quite readily by Classical Theory. It has often been conjectured that especially in the case of diatomic molecules with both electric and magnetic moments parallel to the molecular axis of figure the application of a magnetic field would produce electric as well as magnetic polarization and that *vice versa* an electric field would magnetize the body. This effect has not been observed as yet, though it was carefully looked for with gaseous and with liquid nitrous oxide. To de Haas, in explanation of the failure to observe the effect, is due the suggestion that nitrous oxide molecules are "ambidextrous" and that these left and right-handed molecules are always present in equal numbers. In recent years de Haas has done an enormous amount of work on the superconducting properties of metals. In particular he has studied exhaustively the effect of magnetic fields on superconductors. This is most valuable and the results are likely to lead to very important developments in the theory of electric conduction in metals at low temperatures.

Recently de Haas has succeeded in reaching the lowest temperature as yet attained. It was done through the use of a cooling effect obtained by the adiabatic demagnetization of paramagnetic salts. His latest achievement in this field was to reach a temperature of 0·031° K in a volume of 56 cubic centimetres of potassium chrome alum through demagnetizing the salt when it was thermally insulated.

A ROYAL MEDAL is awarded to Professor SYDNEY CHAPMAN.

Professor Sydney Chapman has made contributions of the highest importance to the kinetic theory of gases. In the original researches of Clerk Maxwell an artificial assumption as to the law of attractive force between the molecules was introduced for the purpose of simplifying the mathematical analysis. Chapman has worked out a generalized theory which assumes no properties for the molecules other than spherical symmetry and has derived formulæ for the viscosity, diffusivity and conductivity of a gas; he has applied these results in detail to three special cases. His investigations have also been extended to the cases of a non-uniform simple monatomic gas and of a composite monatomic gas. Much of Chapman's work has been concerned with terrestrial magnetism. He has outlined a theory of magnetic storms, discussed the energy of such storms and the inferences as to the electric and magnetic state of the interior of the earth which can be deduced from terrestrial magnetic variation. He has developed a general theory of the diurnal variations in the earth's magnetism produced by the moon and sun. The lunar diurnal magnetic variations at many stations have been determined by the analysis of observations extending over many years and the results compared with theory. Somewhat parallel to these investigations have been investigations of the general magnetic field of the sun and of its radial limitation; of solar ultra-violet radiation as a cause of auroræ and magnetic storms, of the influence of solar eclipses on the ionization of the upper atmosphere and the study of the properties of solar streams of corpuscles. Chapman has developed a theory of the lunar atmospheric tide and tested it by the analysis of barometric readings extending over many years at a number of different stations. The composition, ionization and viscosity of the atmosphere at great heights and a theory of upper atmospheric ozone are some of the other problems to which Chapman has devoted his attention.

A ROYAL MEDAL is awarded to Professor EDGAR DOUGLAS ADRIAN for his distinguished work on the physiology of the nervous system. For many years physiologists have been concerned with the changes of electrical potential attending the contraction of a muscle or the propagation of an impulse in a nerve. Adrian's work lies in the natural sequence of these classical researches in electro-physiology, but its great distinction is in the fact that it deals with the activities of the single nerve fibres, single sensory end organs, single muscle fibres, and single nerve cells of which neuro-muscular function is built up. In each of these he has

found one simple quantitative factor in the physiology of sensation or response, namely, the frequency of the rhythmic electrical disturbances which occur in it. Increased intensity of stimulus to a sensory organ means increased frequency of impulses arising from it; increased response from a muscle fibre means increased frequency of stimulation. In this way Adrian's work can be said to have established the "atomic" nature of nervous activity as nothing before has done. Recently Adrian has studied the electrical changes of the cortex of the brain, and with Matthews has shown how, even in conscious man, objective graphic records can be made of the rapid electrical accompaniments of various cerebral states. In earlier work he dealt with the complex effect of light on the retina. In all such respects Adrian has fulfilled the hopes of Keith Lucas's work, whose pupil he is proud to confess himself. Equally he has exhibited and made objective factors in the bodily use of the nerve impulse, which Sherrington recognized but of which he could obtain only indirect evidence. The Society may be satisfied that it has been able thus to honour the inheritor of so great a tradition and one who has himself contributed so greatly to the subject.

The DAVY MEDAL is awarded to Professor WALTER NORMAN HAWORTH.

Walter Norman Haworth is distinguished for his researches on the molecular structure of the carbohydrates. He established, in collaboration with E. L. Hirst, the six-membered oxide ring constitution of the normal simple glycosides, a formulation which is now universally accepted as correct. Following this up, he showed that the more labile so-called γ-glycosides contain a five-membered ring, and he has surveyed the wide field of the saccharides, allotting on experimental grounds a pyranose or a furanose structure to the varied members of the series. Further, he has successfully attacked the problem of the full constitution of disaccharides and even of polysaccharides, and has been able to present a picture of the relations of an entire group of natural products as complete and as satisfying as any in the organic chemist's gallery. His work has a characteristic quality of conclusiveness, due in large measure to a wise insistence on the importance of the use of crystalline reference compounds. In this and other connections he has made notable advances in the appropriate experimental technique.

The DARWIN MEDAL is awarded to Professor ALBERT CHARLES SEWARD.

Professor A. C. Seward has taken a very important part in the great revival of interest in fossil plants which commenced towards the end of

last century and which has provided such a weight of direct evidence for the doctrine of evolution. Of his larger works, the British Museum Catalogues of Jurassic and Wealden plants have been invaluable to subsequent investigators of these floras, while the great text-book on Fossil Plants, published during a period of twenty-one years, has made the wide fields of palæobotany easily accessible to all botanists. This detailed record of the earlier stages of plant history is indispensable alike to the student and the investigator. More recently Professor Seward has collected the results of his long-continued studies in a form appealing to a wider public. An admirable summary of our knowledge of the past history of vegetation, of its distribution throughout the world and of its bearings on the problems of fossil climates and palæogeography is contained in his book "Plant Life through the Ages." In this the fossils are seen as living plants in a real world and the knowledge of them is applied as the key to the significance of the distribution and composition of the flora of the present. More than a hundred memoirs dealing with detailed studies of collections and individual plants have appeared from his pen during this period. Among them his studies on the fossil floras of the southern hemisphere and of the old continent of Gondwanaland must be especially mentioned. His botanical and geological contributions to problems of palæoclimatology give not only the fruits of mature thought, but the mass of data collected is of permanent value for all future investigators. These studies also are of great importance in connection with the theory of Natural Selection.

The SYLVESTER MEDAL is awarded to Earl RUSSELL.

The name of Bertrand, Earl Russell, is proposed for the Sylvester Medal, in recognition of his researches on the Foundations of Mathematics. His earlier writings, the "Essay on the Foundations of Geometry" (1897) and the "Principles of Mathematics" (1903), in the latter of which the attempt was made to reduce all Pure Mathematics to Symbolic Logic, led up to the great undertaking (originally planned as a second volume of the "Principles") of the "Principia Mathematica," the first volume of which (written in collaboration with Professor A. N. Whitehead) appeared in 1910 and two further volumes subsequently. This, the most important work of the "logistic" school (as distinguished from the "axiomatic" school led by Hilbert, and the "intuitionist" school led by Brouwer) is written in a symbolism originally devised by Peano and greatly extended by Russell; its thoroughness may be judged from the fact that it reaches arithmetic only after 666 pages. Most of the original researches published since 1914 by members of the logistic

school have taken the " Principia " as their point of departure; and whether its doctrines eventually win universal acceptance or not, there can be no doubt that it will always rank as a masterpiece.

The HUGHES MEDAL is awarded to Professor KARL MANNE GEORG SIEGBAHN.

Siegbahn began his research work in the field of electro-magnetic waves and published a series of theoretical papers on the transmission of electrical disturbances along cables, and on related electro-magnetic problems. In 1912–1913 he carried out some investigations on the vibrations of telephone-membranes by the use of a method for photographic registration identical in principle with one of the methods nowadays commonly used in the talking-film technique. Since 1914 most of his research work has been devoted to X-ray physics, especially to X-ray spectroscopy. His results in this field are collected in the monographs: " Spektroskopie der Röntgenstrahlen," Springer-Berlin, 1923; Second Edition, 1931, and the " Spectroscopy of X-rays," Clarendon Press, Oxford, 1925. With a view to bridging the gap between the X-rays and the optical spectra, the method with ruled gratings at grazing incidence was made the subject of a thorough investigation by him. In connection therewith two ruling machines of new design were constructed by him and built at his laboratory in Upsala. Gratings ruled on these machines have been successfully used by him and his co-workers for exploring the unknown region of X-ray spectra (20–500 A). He has succeeded in registering and measuring a large number of X-ray series, including the N- and O-series in this region. This work is now in progress and is being pressed forward vigorously. Professor Siegbahn's gratings, though small, are among the finest ever ruled. In addition to his work on long-wave X-rays, Professor Siegbahn is also at present laying the foundations for an exploration of the spectral region between short radio waves and infra-red radiation. One of the most outstanding pieces of work carried out by Professor Siegbahn and his co-workers in the field of radiation includes their beautiful demonstrations of reflection, refraction, interference, and diffraction phenomena with X-rays.

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Fibrillation in the Chick Embryo Heart *in vitro*

I—The Effects of Excess Potassium, Calcium, Magnesium and Sodium, and of High and Low Osmotic Pressures

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(*Communicated by Sir Henry Dale, Sec. R.S.—Received August* 9, 1934—*Revised October* 22, 1934)

1—Introduction

Experiments described in an earlier paper (Murray, 1934) showed that the unco-ordinated contractions which occur when the heart of the young chick embryo is explanted into white of egg are caused by the high potassium content of egg white, aided by calcium and by the low content of sodium. The present paper describes the effects of excess potassium, calcium, magnesium and sodium; experiments on the interrelations between potassium, calcium and sodium; on the effects of high and low osmotic pressures; and on the reversibility of the reaction which results in fibrillation.

In the previous paper the word "fibrillation" was not used, because at that time nothing was known of the mechanism of the phenomenon described, nor whether it bore any but a superficial resemblance to mammalian fibrillation, and the word "twitter" was provisionally used instead. In the paper which follows the present, it is shown that "twitter," while not identical in mechanism with mammalian auricular fibrillation, is a related phenomenon, and the word "fibrillation" replaces "twitter."

2—Methods

The tissue culture method was used. The explants were always cultivated in solid clots as hanging drops on cover-glasses. The medium always consisted of one part of plasma mixed with five parts of a saline. The salines used were all either modifications of the two salines designated in Table I as "Ord PC" and "Sal EW," or else they are fully described in the text. There was always a phosphate buffer. Osmotic pressures were calculated, neglecting the buffer salts and the water of crystal-

lization in the calcium and magnesium chlorides. Clotting of the medium was induced by stirring with a knife dipped in embryo extract; embryo extract was not otherwise used and was thus present only in traces. For reasons given in the previous paper, the medium was not changed, except in the experiments described in Section 8.

TABLE I—(gm %)

	NaCl	KCl	$CaCl_2$ $6H_2O$	$MgCl_2$ $6H_2O$	Osmotic pressure calculated as equivalent to % NaCl
Ordinary Pannett and Compton's solution (Ord PC)..	0·48	0·06	0·03	0·05	0·56
PC (0·76)	0·68	0·06	0·03	0·05	0·76
PC (0·75)	0·67	0·06	0·03	0·05	0·75
Sal EW	0·3	0·252	0·03	0·074	0·54

Except where otherwise stated, the material explanted was entire hearts of chick embryos incubated for two or three days, and having from 20 to 40, usually over 30, somites.

The survival of explants was in general good. The longest survival, as indicated by the continuation of contractile activity, was six weeks, but the average was about four days or a week. Except on certain special questions, explants usually provided the information required of them in the first three days.

No description of the fibrillary movement is here given; it will be found in the succeeding paper.

3—THE ACTION OF EXCESS POTASSIUM

In all the experiments described in this section the fundamental saline used was Sal EW, with varying amounts of KCl. The effects of lower concentrations having been dealt with in the earlier paper, the following concentrations were used in the present work: KCl 0·4%, 0·5%, 0·6%, 0·8%, 1·0%, 1·2%, 1·6%, 1·8%. The numbers of explants made into each medium were: 6, 5, 6, 6, 8, 9, 6, 6.

Co-ordinate Movements—Neither co-ordinate beating of the heart as a whole, nor dissociated beating of independent conus and sinus centres, ever occurred, but, in 0·4% and 0·5%, movements, which appeared to be of a co-ordinate character, occurred on the first day in five explants.

The movement was very weak, was in most explants probably not completely co-ordinated, and was in at least four confined to the conus. Thus a finding of the previous paper, that the beat is more resistant to the action of potassium in the conus than in other regions, was confirmed.

Fibrillation—The results in respect of fibrillation are summarized in Table II. The table shows that: (1) fibrillation could occur in all parts of the heart, and such general distribution was more common, and appeared earlier after explantation, in higher than in lower concentrations. (2) The region most readily thrown into fibrillation was the conus, for fibrillation appeared here immediately after explantation even in the lowest concentrations, only spreading to other regions later. (3) The higher incidence of fibrillation in the higher concentrations of potassium, particularly on the first day, in the sino-atrial and ventricular regions, coupled with the absence of beating from these regions in all the media, shows that the concentration of potassium required to cause fibrillation is higher than that required to paralyse the co-ordinate beat. The ability to contract automatically is thus suppressed by certain concentrations of potassium, but not by lower, nor by a certain range of higher concentrations, though the higher concentrations only allow it in the form of fibrillation. Contractility in response to extrinsic stimuli has not been examined. (4) The amount of fibrillation increased, not only with increasing concentrations of potassium, but also with the passage of time; thus in 0·4% and 0·5% and in 0·6% and 0·8%, there was distinctly more fibrillation, and it was more widely distributed through the hearts, on the second day than at the second examination on the first day, and more then than at the first examination on the first day. This fact indicates either a change in the conditions of the explants, an increased susceptibility to the fibrillating action of potassium, or a change in the medium rendering the action of potassium more effective, and does not invalidate the last conclusion, which applies to fresh hearts immediately after explantation. (5) The fibrillation continues for a number of days, indeed usually until the deaths of the explants, and was thus not a transient phenomenon, as found by Olivo with a different method and with material mainly from later stages.

The statement that fibrillation occurred in some region of the heart does not mean that the whole of that region was necessarily in active fibrillation, but merely that some fibrillation was present. In the table, F means that fibrillation was active through at least a considerable part of the region concerned, f that fibrillation was weak, consisting of sporadic contractions here and there, or was confined to small parts of the region. It was characteristic of this form of fibrillation that it could

TABLE II

Medium	No. of explants	Day 1 1st examination				Day 1 2nd examination				Day 2				Day 3			
		G	SA	V	C	G	SA	V	C	G	SA	V	C	G	SA	V	C
0·4% and 0·5%	11	0	0	0	5 F 5 f	0	2 F 4 f	1 f	3 F 5 f	1	3 F 1 f	2 F 2 f	3 F 4 f	1	2 F 2 f	2 F 3 f	3 F 1 f
0·6% and 0·8%	12	0	2 f	5 f	11+1? F	0	4 f	1 F 3 f	6 F 6 f	2	1 F 3 f	3 F 4 f	8 F 1 f	4	3 f	2 F 1 f	3 F
1·0%	8	4	1 f	2 F 2 f	3 F 1 f	2	3 F 3 f	3 f	6 F	1	2 f	2 f	5 F	1	2 F 2 f	4 f	5 F
1·2%	9	7	1+1? f	1 f	1 F	2	1+1? f	4 f	1 F 4 f	0	0	0	3 f	0	0	0	1 F 1 f
1·6%	6	6	0	0	0	0	0	5 f	4 f	0	0	0	0	0	0	0	0
1·8%	6	6	0	0	0	0	0	1 f	0	0	0	0	0	0	0	0	0

The table shows, for the first three days, the incidence of fibrillation in excess potassium. G, explants in general fibrillation throughout all regions. SA, V, C, explants not in general fibrillation, but showing fibrillation in the sino-atrial, ventricular, or conus regions respectively. F, active fibrillation. f, weak fibrillation. The decreased activity in higher concentrations after day 1, examination 1, is due to the toxic action of these media. The first examination on day 1 was made immediately after explantation, the second examination some hours later.

occur in any area, however large or small, while the remainder of the heart was stationary.

Excess potassium caused paralysis of the heart in diastole; the expanded condition so produced seemed greater than that to which the beating heart normally relaxes. Even the greatest expansions seen did not prevent fibrillation.

4—The Action of Excess Calcium

The solutions in these experiments were all based on ordinary Pannett and Compton's (1924) saline (Ord. PC), the amount of $CaCl_2$ being varied. The hydrated salt was always used. The concentrations of $CaCl_2\,6H_2O$ used were as follows (figures in brackets are the numbers of explants in each medium): 0·3% (8), 0·4% (7), 0·5% (8), 0·6% (8), 0·8% (6), 0·9% (6). These experiments were not quantitatively exact, because unknown amounts of calcium ions were lost from the media by the formation of a precipitate when the phosphate buffer was added and of a further precipitate in the plasma on days succeeding explantation. Nevertheless, since the physiological effects were increased by the addition of increased quantities of $CaCl_2$ to the media, it appears that there must have been increased amounts of calcium in solution. The second precipitate appeared as a granular halo in the plasma around the explants; since it was absent from the plasma remote from the explants its formation was evidently due to an action of the cells.

The Co-ordinate Beat—The effect of excess calcium differed from that of potassium in that, while excess potassium stopped the beat in concentrations generally lower than that required to cause immediate fibrillation in the sino-atrial region and ventricle, in excess calcium, fibrillation and beating frequently occurred together in the same part of the heart. Certain abnormalities of beating were very common. These will be reported as briefly as possible, for they have been studied only incidentally to the fibrillation.

In no concentration used was the beat stopped immediately after explantation, and in the lowest concentrations (0·3% and 0·4%) some sort of beating usually continued throughout the lives of the explants. In concentrations higher than these, beating was very often absent after the first day.

Intermittency of beating was a strongly marked feature, hearts showing alternating periods in which they were beating and in which they were not beating. This occurred in the lowest concentrations used as well as in the higher, and at all times in the lives of the explants, often appearing first immediately after explantation. It tended to occur more frequently

in higher concentrations. The phenomenon is doubtless to be identified with the "periods of Luciani."

Related to intermittency of the whole heart were frequent cases in which, while a constant beat occurred in the sinus or sino-atrial region, the beat in the ventricle and conus was intermittent. This shaded into a condition resembling heart block, for in the less extreme cases the intermissions of beating in ventricle and conus might only be as long as one beat in the sino-atrial region, the result being 2/1 heart block. Similarly, heart blocks of higher grade occurred, such as 3/1 and 4/1, and cases in which the intermissions in the lower parts of the hearts extended over many beats of the higher parts. The extreme condition was one in which there was a continuous beat in the sinus or sinus and atria but none at all in ventricle and conus. This was a point of difference from clinical atrio-ventricular heart block, in which the extreme manifestation is a continued but completely dissociated beating of auricle and ventricle. The block itself might be intermittent. Thus a heart might beat in apparently perfect co-ordination for a number of beats, and then for a period the beat might be confined to the sino-auricular region, or there might be periods of 2/1 or higher block alternating with periods of no block at all.

In the early embryonic heart there is no such sharp morphological separation between atria and ventricle as exists in the adult, for all parts of the heart wall are contractile, and specialized conducting tissues do not yet exist. This was reflected in the less sharp localization of the regions of block, for, while a blocked beat might be stopped abruptly at the atrio-ventricular junction, it might pass beyond this and die out somewhere in the ventricle. The first occurred especially where fairly regular block occurred, such as 2/1, etc., the second probably more often when the ventricle and conus showed long periods of inability to contract. The appearance of the second strongly suggested conduction with a decrement (Drury, 1925; Drury and Andrus, 1924; Drury and Regnier, 1927–29; Drury and Love, 1926; Schmitt, 1928).

In a number of hearts, but not very commonly, the conus developed independence of higher regions and beat either independently of the sinus beat or alone if no sinus beat existed.

Recovery of beat after its loss occurred in several explants in 0·5%, usually towards the end of the life of the explant, and less frequently in other media. It was possible for an apparently normal beat to appear after there had been no beat at all for several days.

In assessing the frequency of beating certain rules were followed: when there was block, or intermittent cessation of lower parts of the

heart, the rate of the sino-atrial region was taken; a few obviously very divergent cases of extremely slow beat were excluded. The average rate of beating in 0·3% was 10 beats in 4·4 seconds, in 0·4% 10 in 4·1 seconds, in 0·5% and 0·6% taken together 10 in 5·5 seconds. In 0·8% and 0·9% such abnormalities as absence and intermittency of beat were too great for any reliable data to be obtained. According to Bogue (1932) the rate of beat of the chick embryo heart *in ovo* is, at 2–3 days, 173 per minute, or 10 beats in 3·5 seconds. In my experience the rate of beats of hearts *in vitro* in a properly balanced medium averages 10 in 4 seconds. It is thus probable that excess calcium has a slight retarding influence on the frequency; at any rate it did not cause acceleration. A point of some interest was that hearts which showed intermittency in its various forms appeared to beat at much the same rate as the more normal hearts. It is possible that a much more intensive study of a larger number of cases might reveal a slight correlation between abnormal behaviour of the beat and fast or slow beating, but no gross and obvious correlation exists. Beating was by no means strictly regular. Three successive counts of 10 beats each of one heart might show a deviation from the average of usually not above 12%.

Fibrillation—The amount of fibrillation was greater, and the time of its onset was earlier, in the higher concentrations than in the lower. This is shown by Table III; for example, fibrillation generally distributed over all parts of the heart never occurred in 0·3%, occurred in only two cases, beginning on the second day, in 0·4%, occurred earlier and more frequently in 0·5% and 0·6%, and occurred in 0·8% and 0·9% in all cases on the first day.

As in excess potassium, while the entire heart might pass into fibrillation, this change might be confined to parts of the heart, and any region, of any size, might be thus affected. Unlike the explants in excess potassium, no region seemed to be especially susceptible to fibrillation, but the ventricle was perhaps the region in which fibrillation occurred least often.

It is well known that excess calcium stops the adult heart in systole; this occurred also in the embryonic heart. Both beating and fibrillation could continue in a heart in which excess calcium had produced a state of partial tonic systole, but it is probable that neither occurred when the tonic contraction was maximal.

5—The Action of Excess Magnesium

The solutions used were, as in the experiments with calcium, all based on Pannett and Compton's salines (Ord PC), the amount of $MgCl_2\ 6H_2O$

TABLE III

Medium	No. of explants	Day 1 1st examination G	SA	V	C	Day 1 2nd examination G	SA	V	C	Day 2 G	SA	V	C	Day 3 G	SA	V	C
0·3%	8	0	0	0	0	0	1 f	2 f	2 f	0	1 F 1 f 2 FB	1 F	1 f	0	2F 1 FB	2 F	1 f
0·4%	7	0	1 F 1 FB	0	3 F 2 f	0	2 F 2 f 1 FB	3 F 2 f	2 F 2 f	2	1 F 1 f	3 F 1 f	1 F 1 f?	2	2 F 1 FB	2 F	1 F
0·5%	8	0	2 FB	1 f	3 F 1 f	2	4 F 1 f	5 F 1 f	1 F 1 f 1 f?	6	1 F	1 F 1 f	1 F 1 f	6	2 F	1 F 1 f	1 F
0·6%	8	0	2 F	1 f?	1 f	3	3 F 1 f	4 F 1 f	1 f 1 f?	5	1 F 1 f	2 f	3 f	4	1 F 1 f	0	1 F 1 f
0·8%	6	4	2 F	0	2 F	6	0	0	0	1	1 f	0	0	1	0	0	0
0·9%	6	4	2 F	1 F	1 F	6	0	0	0	3	3 f	0	0	2	1 F	1 f	1 F

The table shows, for the first three days, the incidence of fibrillation in excess of calcium. G, explants in general fibrillation throughout all regions. SA, V, C, explants not in general fibrillation, but showing fibrillation in the sino-atrial, ventricular or conus regions respectively. F, active fibrillation. f, weak fibrillation. FB, fibrillary beat (see succeeding paper). Fibrillary beat is only recorded when the heart did not also show fibrillation in the same region; this explains its absence from the lower parts of the table. The paucity of records for 0·8% and 0·9% on days 2 and 3 is explained by high mortality in these media.

being varied. The following concentrations were used (figures in brackets are the numbers of explants in each medium): 0·4% (6), 0·6% (5), 0·8% (7 + 6), 1·0% (8 + 6). In 0·8% and 1·0% the first batches (of 7 and 8 explants respectively) revealed infection after about three days, and the experiments were repeated with six explants in each medium.

Magnesium closely resembled calcium in its general effect on the hearts, and will therefore be considered more briefly. It was less active in the induction of fibrillation and caused little if any tonic contraction.

Normal beating hardly ever occurred after the first day, but complete suppression of beating, a common occurrence in excess calcium, was rather rare, and some sort of beating usually persisted until the deaths of the explants. The abnormalities of beating so closely resembled those seen in excess calcium that no further description is necessary. As in excess calcium, the frequency of beating was slightly reduced.

As in its effect on beating, magnesium resembled calcium in producing fibrillation, but differed from it, within the limits of the experiment, in producing it less frequently and in less wide distribution through the heart. It was usually confined to small regions, especially in the earlier days after explantation. Later, it might be more widely distributed, but only very rarely did it involve the entire heart. It could occur in any region, but more frequently in the sino-atrial region than elsewhere. Its relation to the co-ordinate beat was as in excess calcium. It was more readily produced by the higher concentrations of magnesium than by the lower.

The survival of explants in excess magnesium was about the same as in excess potassium and calcium. One explant survived, without change of medium, for the extraordinary period of six weeks, during which it displayed both beating and fibrillation.

6—The Action of Excess Sodium

Excess of sodium was obtained by using, as the saline component of the media, pure solutions of NaCl in concentrations ranging up to the highest in which the explants would survive. As control the " balanced " saline PC (0·76) was used, and the experimental salines were NaCl 0·8%, 1·0%, 1·3%. PC (0·8) is isotonic with NaCl 0·8%. The numbers of explants were: in PC (0·8) 7, in the experimental media, in order of ascending concentrations of NaCl, 7, 8, 8.

Co-ordinate beating occurred in all explants except one in NaCl 1·3%. The normality or otherwise of beating can be expressed in a simple manner as follows: in each medium, the sum is found of the

number of days on which any kind of beating occurred in all the explants, and similarly the sum of the days on which each particular abnormality occurred, such as heart block and intermittency. Then the latter figure is expressed as a percentage of the former.

In Table IV " block " refers to heart block such as occurred so largely in excess of calcium or magnesium. The most interesting fact revealed is the greater amount of abnormality in PC (0·8) than in pure NaCl; the comparison should, of course, be made in particular with NaCl 0·8%, with which PC (0·76) is approximately isotonic. It is evident that

TABLE IV

	PC (0·76)	NaCl 0·8%	NaCl 1·0%	NaCl 1·3%
$\frac{\text{Normal beating}}{\text{Days total beating}}$	$\frac{10}{85} = 11 \cdot 8\%$	$\frac{26}{73} = 35 \cdot 6\%$	$\frac{24}{68} = 35 \cdot 3\%$	$\frac{3}{23} = 13\%$
$\frac{\text{Days block}}{\text{Days total beating}}$	$\frac{22}{85} = 25 \cdot 9\%$	$\frac{11}{73} = 15 \cdot 1\%$	$\frac{15}{68} = 22\%$	$\frac{10\text{–}12}{23} = 47 \cdot 8\%$
$\frac{\text{Days intermittent}}{\text{Days total beating}}$	$\frac{6}{85} = 7\%$	$\frac{4}{73} = 5 \cdot 5\%$	$\frac{7}{68} = 10 \cdot 3\%$	$\frac{4}{23} = 17 \cdot 4\%$

there was no great disparity between the two groups of explants in regard to the length of time during which activity continued, but that the figure for normal beating is higher, and that for block, the chief abnormality, lower, in NaCl 0·8% and 1·0% than in PC (0·76).

Of other abnormalities, there occurred complete dissociation of sinus and conus beats, instances in which the frequency of beating was very irregular, in which occasional beats were dropped altogether, and in which the beat was extremely weak, but block of various kinds was commoner than these. Weakness of beat and short period of survival occurred especially in NaCl 1·3%, probably because of the hypertonicity of the medium.

The frequency of beating was highest in PC (0·76) and decreased with increasing NaCl. In PC (0·76) the average was 10 beats in 5·4 seconds, in NaCl 0·8% 5·9 seconds, in NaCl 1·0% 6·7 seconds, and in NaCl 1·3% 7·9 seconds. Rates of beating were more thoroughly collected in these experiments than in those with other cations, and it emerged that there was no necessary falling off in rate as the age of the explants increased. The regularity of beating was usually imperfect, probably considerably more so than that of the normal beat *in vivo*. Regularity was about equal in PC (0·76) and NaCl 0·8%, but in the higher concentrations of NaCl the beat tended to become more irregular.

Fibrillation never occurred when entire hearts were explanted into these media. It was, however, usual when fragments of hearts were explanted, but was transient and never persisted for more than an hour at room temperature, or less at incubator temperature—39° C. It was always succeeded by beating, which was maintained indefinitely. A brief description will be found in Section 4 of the succeeding paper.

7—The Relation between Potassium, Calcium and Sodium

Olivo (1924) considered that there was an antagonism between potassium and calcium; in his experiments the fibrillation produced by potassium was transitory and was succeeded by paralysis, and this paralysing action of potassium inhibited fibrillation by calcium. The relations between the three cations, sodium, potassium and calcium, were investigated in the following experiments.

Potassium-sodium—Three experiments were performed. In experiments 1 and 2 the media were of different osmotic pressures, but in experiment 3 this possible source of error was eliminated. The media might have been made isotonic by the addition of glucose to the saline of lower osmotic pressure. I thought it wise, however, to avoid this, fearing that the presence of glucose would have an effect on the result; indeed in some early experiments addition of glucose to pure saline media had appeared to be responsible for some anomalous results. The salines used are given in Table V (gm %).

Table V

Experiment	Medium	NaCl	KCl	$CaCl_2$ $6H_2O$	$MgCl_2$ $6H_2O$	No. of explants
1	A	0·64	0·4	0·03	0·074	4
	B	0·48	0·4	0·03	0·074	5
	C	0·3	0·4	0·03	0·074	5
2	A	0·5	0·6	0·03	0·074	7
	B	0·2	0·6	0·03	0·074	7
3	A	0·6	0·6	0·03	0·074	7
	B	0·41	0·82	0·04	0·1	7
	(in ratio	0·3	0·6	0·03	0·074)	

The results are summarized in Table VI.

The table shows that, in all the experiments, the explants in the media containing more sodium fibrillated less than those in which the ratio K/Na was higher. Thus sodium antagonized the production of fibrillation by potassium. It may be added that a little co-ordinate beating

TABLE VI

Experiment	Group	Fibrillation
1	A (NaCl 0·64%)	A little fibrillation almost entirely confined to conus.
	B (NaCl 0·48%)	More fibrillation than in group A, but mainly confined to conus.
	C (NaCl 0·3%)	More fibrillation than in either of the other two groups; usually present in both sino-atrial region and conus and might be present in ventricle.
2	A (NaCl 0·5%)	Fibrillation present in all throughout life, practically always confined to the conus.
	B (NaCl 0·2%)	Fibrillation present in all throughout life, nearly always in both sino-atrial region and conus and frequently in ventricle.
3	A (NaCl 0·6%)	Fibrillation present in all throughout life, usually confined to conus, frequently weak.
	B (NaCl 0·41%)	Fibrillation much more active and more widely distributed than in group A; usually present in sino-atrial region as well as conus, and often in the ventricle.

occurred in group A of experiment 1, less in group B, none in group C. In the other experiments there was no beating.

Calcium-sodium—From the last result it is to be expected that sodium will oppose the production of fibrillation by calcium. An experiment was performed to test this. Three media were used (gm %) :—

TABLE VII

Medium	NaCl	KCl	$CaCl_2$ $6H_2O$	$MgCl_2$ $6H_2O$	No. of explants
A	0·68	0·06	0·4	0·05	6
B	0·48	0·06	0·4	0·05	5
C	0·3	0·06	0·4	0·05	6

The results were positive in that while there was very little beating in medium C, it occurred at some time in all explants in media A and B, and in medium A was present earlier (immediately after explantation) than in medium B (usually not till later on the same day), and was probably in general rather more normal in medium A than in B. Fibrillation occurred in all three groups but was most obvious in medium C. In other media it was largely replaced by beating, but even in medium A it could often be demonstrated by cooling the beating hearts, and it was frequently obvious in hearts that were not beating. The interpretation of this result depended upon the theoretical interpretation of the nature of this form of fibrillation. If, following Olivo, fibrillation be regarded

as not merely normal beating less the co-ordination, but as an activity different in kind from the normal, the result does not necessarily indicate that sodium antagonizes the production of fibrillation by calcium, but merely that it antagonizes the paralysis of the co-ordinate beat. In the paper which follows this, however, it is shown that fibrillation is the result of dissociation of the co-ordinate beat, and therefore the increased beating and decreased fibrillation in higher concentrations of sodium show that sodium does oppose the production of fibrillation by calcium.

Potassium-calcium—Four experiments were performed. Three media were used in each case. The composition of the salines is shown in Table VIII (gm %).

TABLE VIII

Experiment	Medium	NaCl	KCl	$CaCl_2$ $6H_2O$	$MgCl_2$ $6H_2O$	No. of explants
1	K control	0·48	0·4	0·03	—	5
	Experimental	0·48	0·4	0·4	—	6
	Ca control	0·48	0·06	0·4	—	5
2	K control	0·3	0·6	0·03	0·074	6
	Experimental ..	0·3	0·6	0·5	0·074	7
	Ca control	0·3	0·06	0·5	0·074	6
3	K control ..	0·36	0·84	0·036	0·09	6
	(in ratio	0·3	0·7	0·03	0·074)	
	Experimental ..	0·3	0·7	0·5	0·074	6
	Ca control	0·56	0·11	0·935	0·14	6
	(in ratio	0·3	0·06	0·5	0·074)	

Experiment 4 resembled experiment 3 except that minor changes were made in the concentrations of the salts and anhydrous $CaCl_2$ was used instead of the hydrated salt.

In experiments 3 and 4 the osmotic differences between the media, which might vitiate the results of the first two experiments, were eliminated by the same means as were used in the sodium-potassium experiments.

The results of the four experiments are summarized in Table IX.

The table shows that: (1) there was no indication of antagonism between potassium and calcium, in respect of the production of fibrillation, when there was enough calcium present to produce vigorous fibrillation and enough potassium, (*a*) to paralyse the heart (experiment 1); (*b*) to produce a similar vigorous fibrillation (experiments 2, 3 and 4). (2) A sufficient concentration of calcium at least partially antagonized the paralysing action of potassium, so that beating occurred (experiment 1). It may be added that the other well-known antagonisms, by calcium of the expanded state caused by potassium, and by potassium of the tonic contraction caused by calcium, were seen.

8—The Restoration of the Co-ordinate Beat

The following experiments show that fibrillation, whether induced by excess potassium, excess calcium, or by both together, can be "cured" and replaced by normal beating when the medium which induced the fibrillation is replaced by one which is more properly balanced.

Two methods were used. In one, the explants were cut out of the plasma clots and transferred to the new medium by the ordinary method for changing media. The new medium consisted of one part of plasma mixed with five of 0·75% NaCl. In the second method, the explants were left in their original clots, but the cover-glasses carrying the clots were remounted over slides having deep wells filled with 0·75% NaCl; thus the old clots were entirely immersed in a much greater volume of the saline. The saline was usually changed for fresh at least once, in order to wash out as much as possible of the excess potassium or calcium in the clots. The experiments were so conducted as to avoid significant changes of temperature. The second method was found to be the best, giving the same results while avoiding the mechanical injury which was inevitable with the first method.

Table IX

Experiment	Group	Co-ordinate beat	Fibrillation
1	K controls	None	Throughout life, always weak, practically confined to conus.
1	Experimentals and Ca controls	Frequently present usually confined to higher regions.	Throughout life. Distributed over entire heart in all explants.
2	All groups	None	In all explants at some time generally distributed over the entire heart.
3 & 4	All groups	Present immediately after explantation in Ca controls, but disappeared before second day. Did not occur in the other groups.	Began in all explants immediately after explantation and in most was soon distributed over the entire heart.

The experiment was performed upon ten explants in fibrillation caused by excess potassium, upon eleven in which it had been caused by excess calcium, and upon four in which it had been caused by excess of both potassium and calcium. In all explants fibrillation had continued for several days before the experiment was performed. While the details

varied from case to case, the general results may be summarized as follows: fibrillation usually stopped within a minute or two of contact with the new medium and beating began, either immediately or after a lapse of time. In a few hearts, fibrillation, though always immediately and greatly reduced in activity and in the area affected, continued in restricted regions and generally feebly for some time. In one heart, in which fibrillation had originally been induced by both potassium and calcium, it continued for at least two and a half hours, in another, caused to fibrillate by excess calcium, probably for two hours, and in a third, induced by potassium, possibly for three hours. This occasional long continuation of fibrillation is probably to be attributed to the calcium or potassium ions having failed to diffuse away from certain parts of the explant, or to those of the new medium having failed to diffuse in.

The co-ordinate beat usually appeared first in the sino-atrial region, but in the more successful cases it finally involved the entire heart. It was found that potassium fibrillation gave the best results, good " cures " being obtained in five cases, less good in three others, while in two the results were negative. These two were already stationary when the experiment was performed, so both were probably more or less degenerate. Many examples of calcium fibrillation showed only partial recovery of the beat, though fibrillation was always stopped. The reason for this was, probably, that the high concentrations of calcium required to give fibrillation unaccompanied by beating were somewhat toxic, and it was, of course, not permissible to use lower concentrations which would tolerate beating as well as cause fibrillation. Good " cures " were obtained in only three cases, while in six others co-ordinate beating occurred but failed to affect the entire heart. There were two failures, in which no beat appeared. Only one of the four in which fibrillation had been caused by the combined action of both calcium and potassium showed a good " cure," one a rather less complete " cure," while in the other two no beat developed. In all explants of all groups, whether the beat was restored or not, fibrillation was always stopped.

It can be concluded that the reaction, the end result of which is fibrillation, is completely reversible.

9—The Effect of High and Low Osmotic Pressures

It was found in an experiment described in the previous paper (experiment 13) that a balanced medium of sufficiently low osmotic pressure might produce some transitory fibrillation, continuing for a short time immediately after explantation and being then succeeded by beating.

The present experiments show that no osmotic pressure, however high or low, can cause a permanent fibrillation.

Thirteen salines were used. The foundation saline was PC (0·75), and the other twelve salines were of similar composition but with the concentrations of all the salts increased or decreased to give osmotic pressures corresponding to NaCl 0·05%, 0·1%, 0·15%, 0·2%, 0·3%, 1·09%, 1·4%, 1·8%, 2·0%, 2·4%, 3·4%, 3·8%, that is, over a range from very low to very high osmotic pressures. PC (0·75) is isomotic with NaCl 0·75%, and all the other salines are similarly designated, by putting in brackets after " PC " the concentration of NaCl with which each is isotonic. The media were prepared by mixing the salines with plasma in the usual way. Throughout this experiment a heart which had a beat on both first and second days is counted as having had a beat for two days.

The behaviour of explants in PC (0·75) was simple. Beating commenced immediately after explantation and continued in one for eight days, in one for six, in two for four, and in two for three. There was never any fibrillation in this medium.

Six explants were made into each of the hypotonic media. The results may be summarized as follows: immediately after explantation it was found that the beat had stopped but that some fibrillation was occurring in nearly every explant. This continued for a few minutes and then ceased, leaving the heart stationary. The amount of the heart affected by the fibrillation varied greatly, as also did the vigour of the movement. In only one explant, in PC (0·1), was fibrillation distributed actively throughout the heart; in the majority it was present over a larger or smaller part of the sino-atrial region and in many it could be seen also in the conus, but in the latter region it was generally weak. Its occurrence in the ventricle was rare, and when it did exist there it usually seemed to affect only a few scattered cells or else some quite small area. It was probably rather more active and more widely distributed in PC (0·1) than in the other media, but there was no clear difference in this respect. Since it probably never continued longer than 15 minutes and usually stopped in a much shorter time, it was doubtless caused by the shock of the sudden change in osmotic pressure. It is not certain that the fibrillation so produced was identical with that produced by excess of potassium, calcium or magnesium, but there was no obvious difference. The frequency of contraction was perhaps lower, especially in the sino-atrial region. The period of fibrillation was in the great majority of explants, and probably in all, succeeded by a stationary period. In PC (0·05) and (0·1) this was permanent, the explants having evidently

been killed; in PC (0·1) a single exception developed, late on the day of explantation, a weak beat, at first confined to the conus and then to the ventricle and conus, but this had been lost on the following day. In PC (0·15) activity never continued beyond the first day, but four of the six explants developed beating some time after the cessation of fibrillation. In all four the beat was weak, in three exceedingly so, and seemed to be strongest in the lower end of the ventricle, near the conus. The interval which elapsed between the cessation of fibrillation and the recommencement of beating was an hour or more, in one over four hours. In PC (0·2) all the explants displayed beating, which continued in one for fifteen days, in two for two days, in one was confined to the first day; in the remaining two the final cessation of beating occurred after seven days, but it was not continuous over the whole period. There was a decided tendency towards weakness of beating. The duration of the stationary interval varied, but it usually lasted about an hour or rather longer. In PC (0·3) the behaviour of the cultures was in general similar, all developing beats after an interval; the beats were at first weak but became stronger, so that there was little or no indication that the explants were not in a normal medium.

In the hypertonic media, three explants were made in each of the three media: PC (1·09), (1·4), (1·8), (2·0), and six in each of the media: PC (2·4), (3·4), (3·8). Fibrillation never occurred. In the medium PC (1·8), and in media more dilute than this, beating always occurred immediately after explantation and the high osmotic pressure did not seem to have affected greatly the viability of the explants. In PC (2·0) and (2·4), although again beating was always to be seen immediately after explantation, and was at first fairly strong, it tended to become weak, and activity did not continue beyond the second day in PC (2·0) or beyond the first day in PC (2·4). In PC (3·4) and (3·8) the hearts were usually stationary immediately after explantation, then showed weak beating for a short time (not more than one to two hours) and finally became permanently stationary. There was a tendency for the beats to be restricted to the higher parts of the heart, the conus often being stationary.

Summary

When the heart of the young chick embryo was explanted into media containing excess of potassium, calcium or magnesium, a form of fibrillation was induced.

Excess of potassium paralysed the co-ordinate beat, which was more resistant in the conus than elsewhere. The conus was caused to fibrillate

by concentrations lower than those required for other regions, and fibrillation became more widely distributed through the heart, and was produced earlier, in higher than in lower concentrations. The concentration of potassium required to cause fibrillation was in general higher than the concentration required to paralyse the co-ordinate beat.

Excess of calcium frequently tolerated co-ordinate beating as well as causing fibrillation. There were a number of abnormalities of beating and the frequency was slightly reduced. Fibrillation occurred more frequently, earlier, and in wider distribution through the heart, in higher than in lower concentrations of calcium. No region seemed especially susceptible to fibrillation.

The action of excess magnesium in general resembled that of excess calcium, but it produced fibrillation less readily and was more tolerant of beating.

Excess sodium did not cause fibrillation.

Sodium opposed the production of fibrillation by potassium or calcium. In respect of fibrillation, no antagonism could be found between potassium and calcium.

Fibrillation, however induced, could be stopped, and co-ordinate beating restored, by changing the medium for one which was more properly balanced.

Low osmotic pressures caused a transient fibrillation, but permanent fibrillation could not be produced by this means. High osmotic pressures never produced fibrillation.

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Fibrillation in the Chick Embryo Heart *in vitro*
II—The Character and Mechanism of the Fibrillation

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(*Communicated by Sir Henry Dale, Sec. R.S.—Received August* 9, 1934—
Revised October 22, 1934)

1—Introduction

The production of this form of fibrillation, by means of excess potassium, calcium, or magnesium, has been described in the preceding paper. The present paper contains a description of the general character of the fibrillation, of its relation to the co-ordinate beat, and of experiments which lead to a hypothesis of its mechanism.

The methods used were as in the preceding paper, and indeed much of the material was the same, but some was derived from older embryos, and in some the hearts, instead of being explanted entire, were cut into fragments of various sizes. Where it is not stated that this was done, or that the embryos were older than two to three days, it should be assumed that the embryos were of this age and that the hearts were explanted entire. No important difference of behaviour was seen between explants from younger and older embryos.

2—The General Character of the Fibrillation

The outstanding feature of the fibrillation was lack of co-ordination between the contracting cells. So anarchic was the activity that adjacent cells might contract each quite independently of the other, and even different parts of the same cell might show similar independence. Nevertheless, the lack of co-ordination was not absolute. There was a certain tendency for cells to contract in groups. The cells composing a group did not all contract together in the regular manner seen in a co-ordinate beat, but they showed a certain tendency to do so, contracting more or less simultaneously. The general chaos, however, was little affected by this grouping, for changes occurred in the membership of groups, so that a group of cells in a fibrillating sheet might contract once, more or less in co-ordination with one another, and thereafter might redistribute themselves in different groupings or show practically

no co-ordination at all. All that can be said is that while cells might contract independently, they might contract in groups, the memberships of groups might change, and so on. In a very small actively fibrillating fragment of a sino-atrial region about a hundred cells were spread out as a loose network, one cell thick, just beneath the cover-glass. It could be seen that the contraction of any one cell was usually followed by contractions of cells in its neighbourhood, but this co-ordination did not extend more than a short distance through the network. That automaticity existed was shown by cells which contracted when all the cells around them were stationary. Thus the two factors (impaired conduction of impulses and automaticity), which will be put forward below as bringing about fibrillation, were very beautifully demonstrated. The tendency to show some degree of co-ordination seemed greater in calcium than in potassium fibrillation.

It was sometimes possible to observe cells in thin extended sheets, alternately making fibrillary contractions and taking part in co-ordinate beats. In the co-ordinate beat each cell always contracted as a whole and the contraction appeared to consist of a single sharp twitch of the whole cell, and not usually of a wave of contraction propagated over the cell from its point of origin. Too much reliance cannot be placed upon this, for such a propagated wave of contraction might very easily be too rapid for detection in such small cells. Fibrillary contractions frequently differed from those seen in co-ordinate beats in that the cells might become irregularly, instead of evenly and regularly, deformed, and it could be seen that this was due to the contraction being limited to part of the cell, other parts being inactive. In such partial contractions there was clearly no propagation of the mechanical disturbance over the rest of the cell. At the next fibrillary contraction any of three things might happen: the same region might contract again, or the cell might contract as a whole as in a co-ordinate beat, or some other part of the cell might contract. Olivo (1924) described similar intra-cellular disco-ordination. Less commonly, it was clear that the mechanical disturbance was propagated as a wave from one part of the cell to other parts; this was seen especially in large, elongate cells, particularly in explants from the later stages. In one spindle-shaped cell, whose nucleus was about in the middle of its length, and which stretched across a space in the explant and was connected with other cells only at its ends, four different forms of contraction were seen. The contraction usually arose in the neighbourhood of the nucleus and spread thence as two waves to the ends of the cell, but it might spread to only one or other of the two ends, the other half of the cell remaining inactive. Alternatively, the con-

traction wave might start, not in the region of the nucleus, but at one or other of the extremities, and travel either right through the cell to the far extremity, or only as far as the nucleus, the half of the cell remote from the point of origin remaining stationary. It is not improbable that such localized, non-propagated contractions should be compared with the sub-maximal disturbances obtained by Gelfan (1930 *a*, *b*, 1933) in striped muscle fibres.

A feature of the fibrillation produced in the present experiments, which distinguished it from that occurring in the adult mammalian heart, was the localized manner in which it might occur. Thus, it might be present in one atrium and not in the other, or only in part of one atrium, in the tip of the conus and not in its base, along the edge of the convex side of the ventricle and not on its flanks. On the other hand, it might be more widely distributed, especially in higher concentration of the exciting cation, and it was frequently active throughout the entire heart. The region which was least often active was the concave (ant-apical) side of the ventricle, probably because of its slight muscular development.

The amplitude of contraction was shorter in fibrillary than in co-ordinate contractions; this was especially so in potassium fibrillation. In excess calcium, the amplitude frequently appeared longer than in potassium and, though usually short, may sometimes have been as long as in the co-ordinate beat.

To count the frequency of cells in fibrillation was all but impossible, because of the arhythmia and of changes in amplitude and form from one contraction to the next. Mere observation showed that in a heart which had not yet spread, or in unspread parts of hearts from which tissue had emigrated over the cover-glass, the frequency was high compared with that of the intrinsic co-ordinate beat of the same part of the heart, but it was probably a significant observation that when a cell was more or less isolated from its fellows, as in the periphery of a spread region, its frequency seemed less high, and its contractions more regular, than in the case of cells in more complicated relation with neighbouring cells. This suggested strongly that many of the contractions were due to the impact of impulses upon the cell and not to an abnormally high automatic frequency.

The frequency of fibrillary contractions was extremely high in the sino-atrial region (usually much too high to count, however regular it might be), less high in the ventricle, lowest in the conus. This is the order of decreasing frequency of intrinsic co-ordinate beats in these regions (Csaba and Nemeth, 1931; Johnson, 1924; W. H. Lewis, 1924, and personal observations).

Neither co-ordinate nor fibrillary contractions were closely dependent upon any structural relationship of cells to one another, except that two groups of cells could not beat in co-ordination unless there was some organic connection, however roundabout, between them. But both kinds of activity could occur in hearts which had only just been explanted, and in which the histological relations were therefore intact; both could exist in flat sheets of cells joined by their edges or corners and one layer thick, and both could occur in complicated spread-out systems of cells joined in three dimensional networks by threads and trabeculæ of cytoplasm. Although in the last two cases no trace of the histological arrangement of the cells in a normal heart could possibly exist, perfectly co-ordinate beats could nevertheless occur.

3—The Relation between Fibrillation and Beating

In excess calcium or magnesium, beating was able to persist in concentrations of the cation which were sufficient to produce fibrillation. Thus fibrillation and beating occurred simultaneously in the same heart, and even alternately in the same cell, as is shown below. In excess potassium, on the other hand, the beat was usually, if not always, paralysed by a concentration which was below the threshold for the production of fibrillation; hence, these two forms of activity rarely occurred simultaneously in the one heart. The following description of the relations between beating and fibrillation therefore refers only to experiments with excess calcium or magnesium. The effects of these two ions were so similar that statements made of one apply equally to the other, subject to the differences noted in the preceding paper.

Beating and fibrillation were related to one another in two different ways: first, in a form of activity which I call "fibrillary beat" and which is intermediate between beating and fibrillation, and, secondly, in the simultaneous existence in the same tissue of beating and fibrillation.

A. *The Fibrillary Beat*—The fibrillary beat was always, or nearly always, confined to the sino-atrial region, and was identical with the phenomenon called "twitteroid" in an earlier paper (Murray, 1934) but different from that called "co-ordinated twitter." It was a beat in which the co-ordination was imperfect; the cells did not all contract apparently simultaneously, as in a normal beat. This inco-ordination was especially obvious at the beginning of each beat, which was thus preceded by a brief trembling movement, caused by the fibrillary con-

tractions of individual cells. In addition to this, the co-ordination of the cells in the beat itself might be more or less disturbed, and different parts of the sino-atrial region might contract at slightly different times. The analysis to be presented in section 5 shows that the fibrillary contractions of the trembling phase are the immediate cause of the beat which follows, in the sense that the contraction of the first (pace-making) cell in a normal beat is the cause of the beat which follows it.

B. *Alternate Beating and Fibrillation*—Alternation between beating and fibrillation could occur at any place in the heart. The beat differed from the fibrillary beat in being an interruption of a continuous fibrillation, and in that its co-ordination appeared perfect. It is practically certain that the co-ordinate contraction was always a response to an impulse created elsewhere; thus the beat was superposed upon the fibrillating cells, and was not created by them, as in fibrillary beating.

Alternate beating and fibrillation was seen particularly when the frequency of the beat was low, so that the intervals between beats were long, or during the pauses if the beat was intermittent. The occurrence of a beat in a fibrillating region always stopped the fibrillation instantly, but it was resumed again at the next interval of sufficient length. Fibrillation between the beats of a heart, whose beat was neither intermittent nor of low frequency, occurred most readily in the sino-atrial region, less readily in other regions.

It is evident that fibrillation cannot occur between two beats if the interval between them is less than the refractory period of the first beat plus the time occupied by at least one fibrillary contraction. The difference in rate of fibrillation in the different regions was not merely one of frequency of contraction, but of the speed with which the contractions took place; a fibrillary contraction of a cell in the conus was a more leisurely movement than the sharp twitch of an atrial cell. Hence more time was required to permit of fibrillation in the conus than in the sino-atrial region, and therefore, if a conus was beating in co-ordination with a sino-atrial region of high frequency there was less chance of fibrillation appearing in it. Indeed, fibrillation was never seen in the conus under such conditions, but only when there existed long periods between beats or no beats at all. The same applied, in less degree, to the ventricle, in which, the fibrillary movement being faster than in the conus, there was more chance of its appearing. This relationship between frequency of beating and occurrence of fibrillation was demonstrated experimentally by observing the behaviour of hearts during a fall in temperature from that of the incubator to that of the room. Both

the frequency of beating and the rate of fibrillation decreased, and in many cases the beats ceased altogether, to return upon rewarming. Fibrillation generally continued, though slowly, even at room temperature, and as the frequency of beating decreased the occurrence of fibrillation between the beats became more obvious, sometimes appearing in explants in which its existence would hardly have been suspected when warm. It cannot be concluded from this that the normal beat is more sensitive to low temperatures than fibrillation. In the normal beat activity is intermittent, but in fibrillation continuous, and so the longer stationary intervals of the cells are obvious in a normal beat but not in fibrillation, in which the observer sees fewer cells active at any moment.

Two examples will now be given to illustrate the kind of changes seen when a heart, which was showing both beating and fibrillation, was cooled. *Explant* 473, *excess Ca*, *5th day*: when warm, there was intermittent beating, and fibrillation could be detected between beats. Two regions were of special interest. One had an obviously fibrillary beat, while in the other the beats showed a mere suggestion of fibrillary modification. When cooled, the first region lost its co-ordination and lapsed into fibrillation, while in the second the fibrillary contractions became more obvious, so that it resembled the first region when warm. *Explant* 470, *excess Ca*, *2nd day*: when warm, the heart was beating and there was a suggestion in both sino-atrial region and ventricle of an attempt to fibrillate between beats. The beat stopped when the heart was cooled and fibrillation became obvious.

The appearance of many cultures suggested very strongly indeed that the same cell could both fibrillate and beat, for cells, seen to make fibrillary contractions, could also be seen to become shorter and thicker when the co-ordinate beat occurred. This evidence was just short of conclusive, for the contraction in the co-ordinate beat might have been a passive movement due to a release from tension or to the pull of neighbouring cells. Critical evidence was provided by an explant in which certain cells, which fibrillated actively between beats, were so placed that any passive movement caused by other cells taking part in the beat must have made them longer and thinner; they were peripheral cells and the beat tended to pull their central ends away from their points of distal attachment. When the beats occurred they actually became shorter and thicker. This observation was confirmed by several colleagues, who were not told beforehand what they were expected to see.

There was frequently, but not invariably, a perceptible interval after the completion of relaxation from a co-ordinate beat before fibrillary movements were resumed. In one explant in excess magnesium this

interval was of the order of one second, but generally it was shorter than this and was often not detectable at all, the fibrillary movements being resumed immediately. It seems probable that the interval represented the frequency of automatic contractions of the cells.

4—The Behaviour of Very Small Explants

Garrey (1914) found that if a fibrillating adult heart were cut into fragments the continuation of the fibrillary activity depended on the size of the piece, only persisting in the larger pieces, but stopping in the smaller fragments. The latter, nevertheless, retained their irritability and other normal properties.

I repeated Garrey's experiment with the embryonic material, using over fifty hearts. The hearts, which were derived from embryos of various ages (2–3, 4, 5–6 days), were dissected in the usual way and cut into the smallest possible fragments with the aid of cataract knives, under a binocular dissecting microscope. The resulting pieces were mostly of such a size that they could just be seen against a dark background with the naked eye, but some were below this size and some a little larger. Various salines were used in the media and could be classified into four groups: having excess potassium, excess calcium, excess of both calcium and potassium, pure NaCl. The last, whose concentration was either 0·75% or 0·9%, was the control saline; it was shown in section 6 of the preceding paper that such a solution was, when mixed in the usual way with plasma, particularly favourable to the continuation of normal beating in entire hearts.

The explants in the control media showed at first a brief phase of fibrillation, but this disappeared and was followed by co-ordinate beating which was maintained indefinitely. The occurrence of this transient fibrillation has some theoretical importance, and another paper will describe it more fully; at present a brief summary of the observations will suffice. (1) The fibrillary activity closely resembled, in general appearance, that seen in entire hearts explanted into excess of calcium, potassium, or magnesium, though the movement was somewhat less rapid. (2) It appeared immediately or soon after explantation, and was always transient, never lasting more than an hour at room temperature, or for shorter periods at incubator temperature, and it was replaced by co-ordinate beating. Thereafter, it never reappeared, the beating of the explants remaining perfectly co-ordinate. (3) Its occurrence depended upon the explant being a fragment, and it appeared more readily in

small fragments than in large. (4) It could, like calcium and magnesium fibrillation, exist simultaneously with co-ordinate beating, particularly in large fragments. It closely resembled the description given by Fredericq (1929, 1931) of his "microfibrillation."

All the experimental media were chosen because it was known that they would cause fibrillation and fibrillary beating in entire hearts. The same result was obtained with the fragments; in excess potassium, and in excess of both potassium and calcium together the fragments were found to be in fibrillation. In excess calcium the salines used were such as would cause both fibrillation and fibrillary beating in entire hearts, and it was found that some fragments were in more or less fibrillary beat, others in pure fibrillation, just as in different parts of the intact hearts in the same media. The fibrillation caused by the experimental media was not transient, but continued throughout the lives of the explants. It is evident that the fibrillation here described does not depend on the existence of a large mass of tissue.

This result emphasizes the high automaticity of embryonic cardiac tissue. It is well known that such tissue has a higher automaticity than adult cardiac muscle, presumably with the exception of the adult pace-maker. There is general agreement among Cohn and H. A. Murray (1925), Olivo (1926), Bisceglie (1929), and Nordman & Rüther (1931) that the ability of fragments of the heart to contract *in vitro* decreases during the course of development, disappearing first from the ventricle and being finally localized in the right auricle. Little is known of the limits of automaticity at the most highly spontaneous stages; the automatic unit might be the single cell or some kind of cell group. Observations which I have occasionally been able to make strongly support the first view, and show that, if a unit is not constituted by a single cell, a very small number of cells suffices. It occasionally happened that very tiny bodies, which clearly consisted of only very few cells, and sometimes of only one or two, became separated from the main mass of the explant but nevertheless showed active contractions. In the fragmentation experiments, fragments as small as this nearly always died, having been produced by accidental teasing, such fine cutting being of course impossible to the unaided hand. I was, however, able to observe contractile activity in a fragment having not more than six cells, and in slightly larger pieces on several occasions.

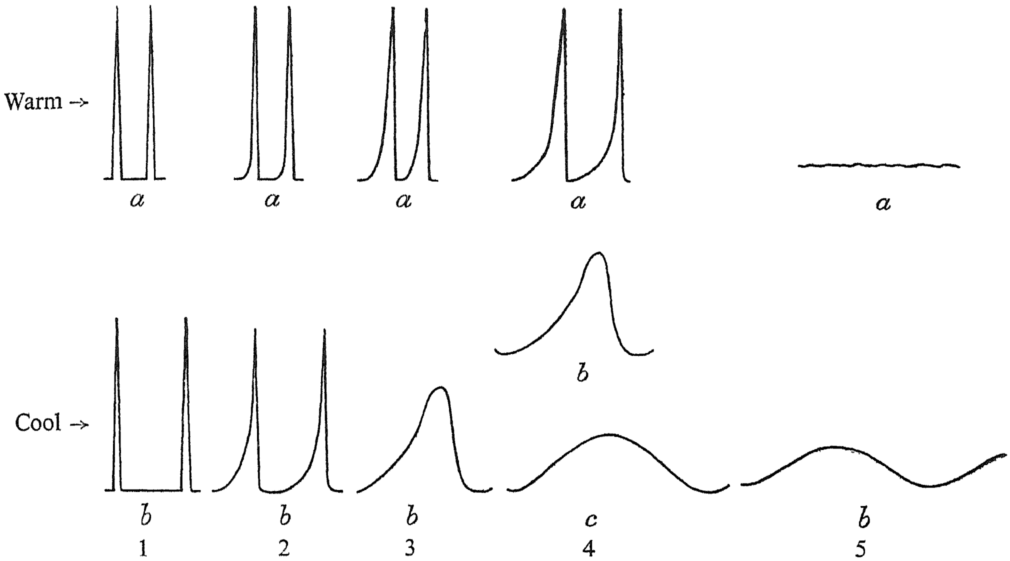
It may be concluded that the automaticity of small fragments of the embryonic heart is great, and that the automatic unit is probably the single cell. Thus, every cell, or every small group of cells, may be regarded as a potential pace-maker.

5—The Effect of Temperature Changes on Small Fragments in Excess Calcium

Many of the explants used in the present section were the same as those used in the last; in other cases, the explants were in similar media but were of larger size. The explants were derived from the sino-atrial region, except when otherwise stated. The experimental media always contained excess calcium; the behaviour of explants in excess potassium is described in the next section. Media containing excess of both cations were not used. Control explants were in media whose saline components were 0·75% or 0·9% NaCl.

If a tiny fragment, invisible or just visible, to the naked eye, which was seen on the warm microscope to be in fibrillary beat, was transferred to a " cold " microscope at room temperature, and watched as it cooled, the first event resembled that which was seen when an entire heart showing fibrillary beating was subjected to the same treatment. At first the explant showed a beat preceded by a tremor. As cooling proceeded, the frequency of beating decreased and the nature of the " tremor " became obvious, revealing itself as a series of fibrillary contractions preceding the contraction of the explant as a whole. It could be seen that the stationary interval between beats was followed by a phase of rapidly increasing activity. First, fibrillary contractions occurred in one or a few cells, then in more and more cells, finally the fragment contracted as a whole, and this was succeeded by an interval when the explant was stationary. If the original fibrillary beating seen on the warm microscope was only slightly fibrillary, so that the beat was nearly normal with only a trace of tremor preceding it, the fibrillary phase of the cycle on the cold microscope was brief, the general contraction following swiftly after a few fibrillary contractions had occurred, and there was usually no further change as cooling proceeded, except a decrease of frequency and some slight extension of the fibrillary phase, fig. 2. But when the co-ordination of the original fibrillary beat on the warm microscope was poor and the fibrillary phase obvious, the explant on cooling passed through the above described condition and proceeded to another. In this, fig. 4 *c*, the simultaneity of contractions further decreased and the cycle, instead of being: stationary—fibrillary contractions—beat as a whole—stationary, lost the phase of beating as a whole. The cycle then became: stationary—fibrillary contractions of increasing numbers of cells, rising to a maximum—fibrillary contractions of decreasing numbers of cells, falling to a minimum—stationary. Thus in such an explant cooling produced a disintegration of the beat, which became

a cyclic fibrillation. This term is used to indicate a fibrillation showing cycles of rising and falling activity, and bears no relation to the question of a circulating impulse. The difference between high activity and low activity was in the numbers of cells engaged in contraction or relaxation at any moment. In a slightly more extreme form the disintegration proceeded so far that the stationary intervals ceased to exist, and there was therefore uninterrupted fibrillation which, however, remained cyclic, there being periodic maxima and minima of activity. These three



FIGS. 1–5—Diagrams illustrating events when small fragments in fibrillary beat or fibrillation are cooled. Ordinates represent number of cells active, abscissæ represent time. In each figure, *a* represents condition when warm, *b* or *c* when cool. In fig. 4 two stages of cooling *b* and *c* are shown. In figs. 3–5, *b* and *c*, only one complete cycle is shown in each. Fig. 1 normal beat, figs. 2–4 fibrillary beats with increasing degrees of fibrillary modification, fig. 5 fibrillation becoming periodic on cooling. Fibrillation not showing periodicity might be represented by fig. 5 *a* both before and after cooling.

conditions, described in the order of decreasing co-ordination in the explants, were connected by all intermediate conditions. For example, fig. 3, a fibrillary beat which when warm showed a degree of co-ordination intermediate between the first and second of the above examples, would when cool show a fibrillary phase, a phase of maximal activity sufficiently well co-ordinated to be called a beat, but suggesting by the small amplitude of contraction that many of the cells were not taking part, and then a second phase of decreasing fibrillary activity. This might be followed by a stationary interval of brief duration, or might sink to a minimum

of activity and then pass directly into the rising activity of the first phase of the next cycle. Since it has not yet been possible to obtain graphic records of these changes, they are illustrated by quite imaginary curves indicating the general form of the visible cycles of activity. Ordinates indicate number of cells contracting, and abscissæ represent time.

Similar small fragments, engaged when warm in active "pure" fibrillation with no visible sign of co-ordination, fig. 5, when cooled generally behaved in a manner very similar to that seen in pieces originally in fibrillary beat. Continuous fibrillation was maintained for a few seconds, but was succeeded by periodic activity in which cycles were displayed resembling those shown in figs. 4 *c* and 5 *b*; that is, an uninterrupted fibrillation with periodic maxima and minima. As cooling proceeded this condition might be maintained, or the difference in activity between maxima and minima might decrease or vanish, leaving, when fully cooled to room temperature, a continuous weak fibrillation of more or less unchanging activity. It was unusual for any clear stationary period to intervene between cycles at any stage of cooling. Less commonly, such an explant might on cooling pass into a condition resembling fig. 3 *b*, having a fibrillary phase leading up to a more or less co-ordinated beat of the piece as a whole, usually followed by a brief second phase of fibrillation, with or without a stationary interval between this and the next cycle. This was often not the final condition, the piece ultimately passing into cyclic fibrillation. A certain number of explants, in fibrillation when warm, failed to show any periodicity when cooled, retaining a slow but unchanging fibrillation. The number of such pieces in a set of explants tended to increase as time went on; for just as in entire hearts fibrillation tended to increase, and beating to decrease, as day succeeded day after explantation, so small pieces which at first showed cycles on cooling might later show none.

A question not without importance as bearing on the larger problem of the part played by the refractory period in this form of fibrillation, is whether or not the same cell contracted more than once in the same cycle. This sometimes occurred, but was probably not usual. Cells could only rarely be seen to contract twice and, in fragments showing fibrillary beat, the amplitude of the beat diminished with the co-ordination, suggesting that cells which had already contracted in the fibrillary phase did not do so again in the co-ordinate phase. Sometimes, however, observation left little doubt that one cell could contract twice within a cycle, especially if the co-ordination was poor, or in large fragments.

The duration of each cycle varied greatly in different explants and probably from cycle to cycle in the same explant, but was of the order

of seconds. Thus, the durations of six cycles displayed by a cooled explant, which when warm was in active continuous fibrillation, were measured as follows : 6·9, 4, 5·5, 8, 4·3, 5·2 seconds, and the stationary intervals between another series lasted : 4·5, 4·3, 2·7, 1·9, 3·5 seconds. Another fragment, which when warm was in fibrillary beat with poor co-ordination, showed, when cool, cycles measuring 1·2, 1·2, 2, 1·7 seconds. Such measurements can only be taken as indicating orders of magnitude, for the shorter cycles were too brief for accurate measurement and in the longer ones there was frequently great difficulty in knowing precisely when one cycle ended and another began. It was clear that the duration of each cycle depended on the degree of co-ordination and to some extent on the size of the explant. When the co-ordination was high, so that the preliminary fibrillation was rapidly succeeded by a beat, figs. 2 and 3, the duration was brief, because most of the cells contracted together in the beat phase, but if co-ordination was poor, activity merely rising to a maximum and falling again, figs. 4 and 5, the cycles were long.

Similar explants in a control medium beat normally both when warm and when cool, the beat being always a sharp contraction of the whole piece, divisible only into the two phases of contraction and relaxation, and all the cells moving in perfect co-ordination, fig. 1. The duration of each beat was too brief for measurement

These observations show that fibrillation did not consist of entirely unco-ordinated automatic contractions, but that an impulse was present. If no impulse had existed, explants fibrillating when warm would have shown no periodical variations when cool, but merely a slowing of continuous fibrillary contractions.

In further interpretation the normal beat, the fibrillary beat, and fibrillation will be considered separately.

(1) *The Normal Beat*—The characteristic of normal beating, which distinguishes it from fibrillary beating and fibrillation, is the apparently simultaneous contraction of all the cells. This is, of course, only apparent, there being in reality a wave of contraction which passes so rapidly over the explant that it cannot be detected by the eye. The cycle of activity is very brief, and cooling does not appreciably prolong it.

(2) *The Fibrillary Beat*—In fibrillary beating, the co-ordinate phase, the co-ordination of which may itself be more or less impaired, is preceded by fibrillary contractions. The first of these contractions corresponds with the contraction of the first cell in normal beating, and the impulse set up by it would normally cause an immediate beat. But

since the cycle consists of increasing numbers of fibrillary contractions only then followed by the beat, clearly the passage of the impulse over the explant is slow. As in the normal beat, the impulse spreads outwards from the first contracting cell, but it spreads relatively slowly, affecting more and more cells as it proceeds, until, by geometrical progression, enough cells are simultaneously activated to give the beat phase. If the impulse is able to affect all the non-refractory cells at once in the beat phase, this will not be followed by any second fibrillary phase, but if it fails to do this, so that some cells are still ready to contract when the majority have taken part in the first fibrillary phase and in the beat, the impulse will then spread to them and a second fibrillary phase will result. The phenomenon of slow travel of the impulse will in what follows be ascribed to impaired conductivity, for the present without prejudice to the question whether it is due to prolonged refractory periods or to depressed cell to cell transmission.

(3) *Fibrillation*—Fibrillation differs from fibrillary beating in that activity is continuous, there being no stationary periods, but it shows itself to be based on the same mechanism as the fibrillary beat by its exhibition of periodicity when small fragments are cooled. Its behaviour in these experiments differs from that of fragments in fibrillary beat in the greater length of the cycles, usually without any beat phase, and at least often without any stationary period. The greater duration of the cycle is connected with the absence of the beat phase and clearly means that the impulse traverses the explant more slowly. Thus fibrillation is a more extreme manifestation of the same condition as is responsible for fibrillary beating, *i.e.*, impaired conductivity. Fibrillary beating, however, shows periodicity (alternating active and inactive periods) when warm as well as when cool, which fibrillation does not. The explanation of this is to be found in the greater length of the active cycles in fibrillation. The first contraction in each cycle in cyclic fibrillation corresponds with the first contraction of the fibrillary phase in a fibrillary beat and with the first contraction in a normal beat. The frequency with which new cycles are initiated is therefore determined, in all three forms of activity, by whatever cell in the explant has the highest automatic frequency. The intervals between successive automatic contractions of this cell are occupied by (*a*) the period when the explant is active, and (*b*) the resting period. If the active period is prolonged the resting period is reduced. Thus, if the active period is sufficiently prolonged it will occupy the whole of the resting period, and activity will still be in progress when the moment arrives at which the new cycle

is due to begin. This is possible because in fibrillary and normal beating in excess of calcium or magnesium the frequency of beating is probably slightly, but not greatly, reduced (see the preceding paper). Thus the length of the resting periods is not increased in proportion with the active periods. The cells which contract first in one cycle will contract automatically again before the end of the cycle, so originating a new cycle, and the result is a fusion of cycles, or no cycles at all. If one studies a number of small fibrillating explants on a warm microscope, one can find specimens which show no periodicity, others in which a slight hint of periodicity can just be detected, and a series of transitions leading to the more advanced cases of fibrillary beat with two fibrillary phases and brief stationary intervals.

If this were the whole mechanism it should have been possible to see a wave of contraction, starting at some point, spread out over the rest of the explant, but no such wave could usually be seen. The increasingly large number of contracting cells did not seem to have any regular topographical relation to one another, but to be scattered in a somewhat haphazard manner over the piece of tissue. Now, since the explants studied in these experiments were all very small, the automatic frequencies of the cells within each fragment must have been very nearly the same, while the rate of travel of the impulse was very greatly reduced. It is therefore to be expected that although the pace-making cell (A), which starts the cycle, sends out an impulse, other cells (B, C), whose automatic frequencies are slightly below that of A, will be able to execute automatic contractions, and themselves set up impulses, before the impulse derived from A can reach them. Thus in each cycle, and provided the piece is not too small, instead of a single impulse travelling slowly through the explant, there will be several, or even many, originated at different points, and at slightly different times. It is of interest in this connection that Rothberger and Winterberg (1911) found in adult mammals that injections of barium, and to a lesser extent of calcium, chloride were able to cause the assumption of automatism by ectopic pace-makers in the ventricle. It is possible that the presence of such multiple pace-makers may be an important factor in the fibrillary phase of the fibrillary beat, a number of cells performing automatic contractions and setting up a number of impulses which, since they originate very nearly simultaneously, show addition of their effects in the production of the co-ordinate phase. If this, rather than the slow spread of a single impulse, is what actually happens in fibrillary beating, it does not greatly change the description of the mechanism, for multiple pace-makers depend upon slow spreading of the primary impulse, and so upon impaired conductivity.

Such secondary impulses will have opposite effects on the duration of the cycles according to the size of the piece. Below a critical size, their occurrence will be less probable, but if they do occur they will tend to shorten the cycle, as hastening the moment when all the cells will be refractory. Above the critical size, the large number of cells will increase the probability of the occurrence of secondary impulses, (*a*) because the primary impulse must travel further before it has affected the whole explant, (*b*) because there are more cells, any of which may set up impulses, and (*c*) because the large mass of tissue makes it possible for two or more impulses to exist in the same fragment, without ever putting all the cells into the refractory state. Thus there would be no periodicity even when cool. It was, in fact, found that large fragments which were in fibrillation when warm showed periodicity when cool less readily than small pieces, and entire hearts in pure fibrillation never showed any periodicity at all. If a large explant had a fibrillary beat when warm, it showed cycles of activity when cool, for the existence of the fibrillary beat demonstrates that the conductivity was high enough to enable the first impulse, or a series of impulses taking origin within a very short period of time, to affect the whole explant practically at once, so putting all the cells into the refractory state.

In very tiny pieces, the formation of secondary impulses should be rare, because of the small numbers of cells, and because of the short distances which the primary impulse has to travel. Thus such explants should always show periodicity on cooling, and it might sometimes be possible to see the course taken by the impulse. The preparation of such tiny explants was difficult, for they generally died, killed by the rough handling which they received. A few were obtained and studied. If the fragment were sufficiently small continuous fibrillation seemed not to exist, there being always stationary intervals. In the smallest piece I have been able to study in the present connection the impulse could be seen to arise at one end of the explant and travel to the other end. The explant consisted of probably three or four, and of not more than six cells. When warm, there was a beat which appeared co-ordinate but showed an indication of disco-ordination in that the form of the contraction differed from beat to beat. When cooled, it merely slowed; with so few cells there could be no gradual rise and fall of activity. A second, slightly larger fragment, when warm had a sort of beat in which disco-ordination was more obvious than in the first fragment, and when cooled exhibited cyclic fibrillation with definite stationary intervals, but with no clear gradual rise and fall of activity. A third and still larger piece, estimated to consist of between twenty and thirty cells, showed

when *warm* an irregular cyclic fibrillation, with no beat of the piece as a whole but with intervals when it was stationary, or when activity was at a minimum. These " intervals " were mere points of time, not long or clear enough to render possible the measurement of the frequency of the cycles. When cooled, the explant showed cyclic fibrillation with rather long periods of activity separated by quite definite and measurable stationary intervals and with no real rise and fall of activity. Thus, so far as the available facts go, they are in agreement with expectation from theory.

Summarizing, three factors co-operate in controlling the occurrence of fibrillation: impaired conductivity, frequency of automatic contraction, and the number of cells present. These factors act together in the following manner: the length of the active cycles is determined by (*a*) the rate at which the impulse traverses the explant, that is, by the conductivity; (*b*) by the size of the explant, since manifestly the impulse will take longer to traverse a large than a small explant. Whether successive cycles will fuse, as in fibrillation, or remain separate, as in fibrillary and normal beating, is determined by (*a*) the lengths of the intervals between the initiations of successive cycles, that is, by the frequency of automatic contraction; (*b*) by the durations of the cycles. Whether or not there will be secondary automatic contractions and secondary impulses is determined by (*a*) the frequency of automatic contractions; (*b*) the size of the explant (number of potential pacemakers and amount of room available to impulses); and by (*c*) the rate at which the primary impulse traverses the piece (conductivity).

It remains to describe events when cooling was succeeded by rewarming. Fragments which were at first in fibrillary beat showed a return to the original condition. Pieces originally in pure fibrillation, but which showed periodicity when cooled, might on rewarming behave in either of two ways, fig. 6. Usually there was some gain in co-ordination, compared with the original condition, the periodicity which was developed on cooling being retained during rewarming, sometimes even to the extent of developing a true fibrillary beat, in which each cycle ended in a more or less general contraction of the explant as a whole. This condition was, however, unstable, and the piece sooner or later reverted to fibrillation. The following interpretation is offered. Warming raises both the automatic frequency and the conductivity. The increased conductivity tends to shorten the cycles, increasing the intervals between them, and making each cycle more and more like a beat. The higher frequency, on the other hand, reduces the intervals between cycles, so tending to make them fuse. If the two factors are raised about equally,

the cycles will remain separate and the shortening of each cycle may produce a fibrillary beat. But since conductivity is impaired by the excess calcium, its upper limit is set lower than normal, while the frequency suffers no such disadvantage; therefore, as warming proceeds, a stage is reached at which new cycles originate before the end of the old, and fibrillation follows, fig. 6, *a–d*.

In the second mode of behaviour, which occurred in probably fewer explants, the periodicity seen on cooling was more rapidly lost on warming. The frequency of cycles increased more rapidly than the individual cycles shortened, so that successive cycles became indistinguishable before each was able to establish any semblance of beat, fig. 6, *a*, *b*, *e*.

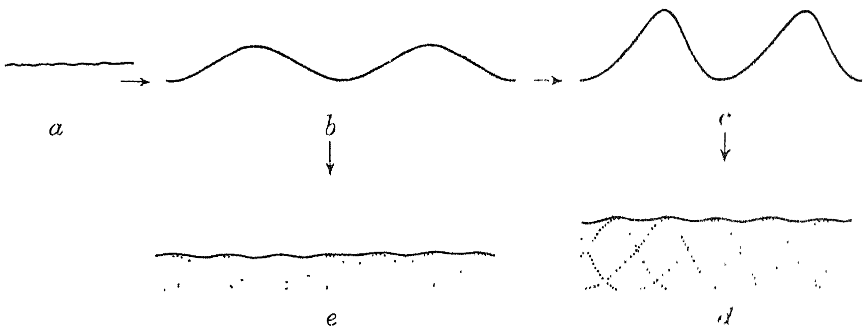


FIG. 6—Diagram representing behaviour of small fragment in fibrillation, on cooling and rewarming. (*a*) Condition when warm; (*b*) cyclic fibrillation on cooling; (*c*) increased co-ordination during rewarming; (*d*) collapse of co-ordination, and return to fibrillation, as warming continues. The dotted curves indicate individual cycles which have fused. (*e*) Resembles (*d*), but fusion of cycles occurs without the recovery of co-ordination seen in (*c*). Ordinates : number of cells active. Abscissæ : time.

This fusion of cycles amounted to fibrillation. It is evident that in these cases the upper limit of conductivity was set lower than in the last. Explants originally in pure fibrillation, but which on cooling showed no periodicity, when rewarmed usually simply returned to the original condition, by acceleration. It is inferred that in such explants conductivity was so low that cooling failed to separate the cycles. Less often, the rewarming caused the appearance of the periodicity which had been absent during cooling, even to the extent of producing fibrillary beats. The interpretation of this is difficult. The facts show that, while conductivity is not so depressed that the cycles can never be separated, yet its depression is severe enough to make the exhibition of periodicity difficult. It may be supposed that the chaos of fibrillation is too complex for resolution into cycles by simple cooling, but that the mere deceleration

so reduces the number of active (and refractory) cells at any moment that, when the frequency and automaticity are again slightly raised, some cell is able to produce an impulse which can traverse the whole piece before the system is complicated by automatic contraction of other cells. This is then repeated for a time, but the beat is unstable and collapses by the same mechanism as in other cases.

Recovery of co-ordination on rewarming, and also during cooling, was particularly well seen in large fragments of the ventricle, or in the entire explanted ventricles of 2–3 day embryos. Assuming that such explants were originally in pure fibrillation, they might behave in four ways. (*a*) The explant might pass into fibrillary beating, for this is more readily caused than in similar-sized pieces of the sino-atrial region. The lower automatic frequency of the ventricle makes the exhibition of periodicity easier than in sino-atrial tissue, for both the frequency of cycles and the number of subsidiary cycles must be lower. (*b*) Beating might not occur at all. (*c*) Rewarming might cause alternating periods of beating and of fibrillation, but as this (*d*) could also occur without change of temperature, any explanation must cover the latter condition. The process observed in (*c*) and (*d*) was totally different from that seen in small fragments when cooled or warmed, and was much more complicated. The explants, in continuous fibrillation without detectable periodicity, were seen to be developing some degree of co-ordination; this waxed and waned, pure fibrillation frequently reappearing after some slight co-ordination had been gained. Gradually the degree of co-ordination and the amplitude of the partially co-ordinated contractions increased, resembling at first a very fibrillary beat, and then, quite suddenly, over two or three beats, the unco-ordinated fibrillary contractions ceased, the amplitude of the beats increased greatly, and the entire explant was beating normally. This normal beating continued for varying periods, but its end was always the same: a sudden drop in amplitude and reappearance of fibrillary contractions, which increased so rapidly that, in the course of two or three beats, all co-ordination had disappeared and the explant was back in pure fibrillation. In one case the collapse was not quite complete, a little very imperfect co-ordination remaining. The fibrillation which followed the collapse of the beat was an unusually rapid movement, which gradually slowed down to the normal rate. The whole process might then be repeated all over again, an indefinite number of times. It was seen in four fragments of three hearts without any change of temperature, and it is this which renders interpretation difficult; for any hypothesis must provide an intrinsic mechanism. It is not impossible to imagine one which would

establish beating in the manner observed, but it is difficult to account for the sudden relapse into fibrillation. As the phenomenon has only been observed in a few explants it seems better to defer any attempt at explanation until more information has been obtained.

It may be added that it was occasionally possible to induce beating in entire hearts and in such large pieces as the entire sino-atrial region by first cooling the explants and then rewarming. It is not known whether the preliminary cooling is essential. The beat usually collapsed immediately after removal from the source of heat. These cases were difficult to study but it may be supposed that the heating, which was probably to above incubator temperature—39° C—so accelerated conduction that there was some separation of cycles. In entire hearts the recovered beat was always confined to the sino-atrial or conus regions, and was never a beat of the entire heart.

Relations between Dominant and Subordinate Regions—It was stated in an earlier section that the fibrillary beat was always, or nearly always, confined to the sino-atrial region of entire explanted hearts. But fibrillary beating did occur in the ventricle, or fragments of ventricle, isolated from the rest of the heart. The reason for this is simple. In an entire heart, the low automatic frequency of the ventricle and its large size make it impossible for a fibrillary beat to occur before the ventricle is caused to beat by the arrival of an impulse from the sino-atrial region. If the concentration of calcium is sufficiently high to prevent beating in the sino-atrial region, either it has the same effect on the ventricle, or, if not, the ventricle is thrown into fibrillation by the chaotic impulses arriving from the sino-atrial region. Thus the ventricle is never able to display a fibrillary beat in an entire heart. In some explants of fragments of heart these relationships were very beautifully demonstrated. One explant was particularly instructive. This was one of nine strips into which an auricle of a 5½-day embryo had been cut. The whole piece showed, on the warm microscope, a fibrillary beat, and one end was identified as the pace-maker, for the beat could be seen to originate there. The explant was warmed to above incubator temperature and was watched while it cooled to room temperature. The behaviour of subordinate regions clearly depended on that of the pace-maker. At first this was in rapid fibrillary beat, while the subordinate part beat in co-ordination with it but showed practically no fibrillary modification. These relations resemble those between the sino-atrial and ventricular regions in many entire hearts at incubator temperature. As the temperature fell, the duration of the fibrillary phase in the beats of the pace-

maker of course increased, and the beats of the subordinate region became fibrillary too. For this two factors must have been responsible: the partial disintegration of the impulse from the pace-maker and the increased time allowed for automatic contractions in the subordinate regions. Next, as the beat in the pace-maker became more fibrillary, the subordinate region lost its co-ordinate phase and became a cyclic fibrillation, the cycles being in accord with those of the pace-maker, and finally the pace-maker itself passed into cyclic fibrillation, and the periodicity practically disappeared from the subordinate region, which was thus in continuous, non-cyclic fibrillation. These later changes are evidently to be interpreted as a continuation of the earlier, the co-ordination in the subordinate region being broken up by the further disintegration of the impulse and the more frequent occurrence of subsidiary automatic contractions. Thus, for fibrillary beating to occur in subordinate regions, the impulse must not be so frequent as to prevent automatic contractions, and must not be so disintegrated as to make beating in the subordinate region impossible.

6—Periodicity in Excess Potassium

The absence of beating from entire hearts and fragments when explanted into excess potassium suggested that the fibrillation which then occurred might be due entirely to automatic contractions, impulse transmission being completely suppressed. The contrary was, however, rendered probable by the observation that adjacent cells in fibrillating sheets seemed to show a tendency to contract together, and by the fact that the frequency of fibrillary contractions was higher than that of normal beating and probably not below that seen in excess calcium. Examination of tiny fragments of hearts fibrillating in excess potassium showed, during cooling, that cycles of increasing and decreasing activity could be detected, but were very much less obvious than in excess calcium. That such cycles did exist was confirmed by two colleagues to whom I showed a preparation, without telling them whether I could see periodicity or not. In slightly larger fragments, which would show clear periodicity in excess calcium, cycles could not usually be seen, but if the eye were fixed upon some chosen region in such an explant it could be seen that the cells tended to be active together and stationary together. No recovery of the beat was ever seen on cooling or warming. It is concluded that the mechanism of potassium fibrillation resembles that of calcium fibrillation, but that the impairment of conductivity and the part played by automatic contractions are greater.

7—Discussion

The decreased rate of travel of the impulse, accepted in the preceding section as the principal factor in causing fibrillation, might itself be due to (*a*) the presence of islands of refractory cells causing the impulse to travel circuitously, (*b*) to a depression of the rate of transmission of the impulse from cell to cell, (*c*) to decrement in conduction, so that impulses would not only travel slowly, but would die out after they had travelled some distance from the starting-point.

(*a*) Islands of refractory tissue doubtless exist in an explant showing fully established fibrillation, for they would be brought into existence in the wake of every little contraction wave in the whole chaos. But the decreased rate of conduction cannot have been originally caused by them, unless either the rate of beating was so rapid that each impulse became involved in the partial refractory period of the one preceding it or, with unchanged rate of beating, the refractory periods became so prolonged that some cells were still refractory at the ends of stationary intervals. The first alternative must be excluded because excess calcium and magnesium reduce rather than accelerate the rate of beating, and potassium stops it. The second, requiring refractory periods extending over the interval between two beats, would not permit contractions of higher frequency than the normal beat, but the frequency of contraction in fibrillating sino-atrial, and almost certainly ventricular, cells is decidedly higher than this, so the refractory periods cannot be of the lengths required.

To decide between (*b*) and (*c*) is impossible, but (*c*) seems to accord with the facts rather more readily than (*b*), for if impulses actually die out before they have traversed the whole of the available tissue, the opportunity afforded for the activity of multiple pace-makers is evidently increased. The form of block seen in excess calcium and magnesium, in which the beat is not stopped abruptly at the atrio-ventricular junction, but dies out somewhere in the ventricle, is perhaps evidence in favour of decremental conduction.

Thus the slow rate of travel of the impulse is due to some kind of direct impairment of conduction, whether decremental or not. Independent evidence that conduction can suffer decrement, and the impulse die out, has been provided by Drury and his colleagues (1925, 1924, 1927–29, 1926), and Schmitt (1928) found that excess potassium could produce this effect. Daly and Clark (1921) found that intraventricular conduction in the frog's heart was adversely affected when the $CaCl_2$ in the perfusate was increased to 0·048%, when the KCl was increased

to 0·064% or reduced to 0·004%, and when the NaCl was reduced to 0·16%, Seliskar (1926) found that intra-auricular conduction in the tortoise was diminished by excess potassium and Schellong (1924–25) found that conductivity was impaired, along with the other functions of "excitability" by excess potassium. Wiggers and co-workers (1930) attributed potassium fibrillation in the mammalian ventricle to depressed conduction and MacWilliam (1917–1919) cites potassium among other agencies as a cause of depressed conductivity.

Schellong, in a series of papers (1924–5), and also other authors, brought evidence to show that the excitation process and its transmission are not separable cardiac functions, but aspects of the single fundamental function, excitability. Now, the facts described in the present paper leave no doubt that the transmission of the impulse is in some way impaired, and it might therefore be expected that the frequency of the automatic contractions would be reduced in proportion. Such an expectation, however, rests on the assumption that automatic frequency is an expression of excitability, which is not known. Further, the frequency of beating can vary without change in the rate of conduction, when the vagus (Lewis, Drury, and Bulger, 1921) or accelerans (Izquierdo, 1930) is stimulated; thus rate of beating can vary independently of conductivity. In the present experiments, firstly, the fusion of cycles, which is revealed in the cooling and rewarming experiments, could not happen if the frequency were reduced in proportion with the extension of the active periods, and secondly, in a medium containing 0·4% $CaCl_2$ $6H_2O$, which tolerates both beating and fibrillation, the frequency averaged ten beats in 4·1 seconds, which may be compared with 4 seconds in more properly balanced media (see preceding paper), a difference well within the ranges of variation and of error.

An argument which might be raised against depressed conductivity as the cause of the embryonic fibrillation is that a co-ordinate beat, originating in the pace-maker, can sweep across subordinate fibrillating regions. If the fibrillation has been caused by depressed conductivity, it is not at once obvious why a co-ordinate beat should be possible. An explant in pure fibrillation has (if the temperature remains constant) considerable difficulty in initiating a co-ordinate beat; this is illustrated by the lengthy integrative period in the recovery of beating by isolated fibrillating fragments of ventricles, but if the ventricle is in connection with a sino-atrial region which has a beat, fibrillary or not, it responds fairly readily to the impulse and shows itself able to beat. The explanation lies in the different degrees of difficulty inherent in creating a co-ordinate impulse, and in responding to such an impulse already created.

When such an impulse already exists, created elsewhere, it advances into the fibrillating tissue on a wide front, and instantly stimulates all non-refractory cells to beat. But when an impulse is being created, it starts from only one or a few cells, and so is unable to exert the influence which is easily attained by a fully developed impulse arriving from elsewhere.

It is next of interest to decide whether the fibrillation described in the present paper is identical in nature with mammalian fibrillation. Between the two phenomena there is a superficial resemblance, for, in both, the outstanding features are the lack of co-ordination and the high frequency of contractions. But there are also differences: the occurrence of beating and fibrillation together in the same tissue, the frequent restriction of fibrillation to small localized areas, and its continuation in tiny fragments of the heart, are all characteristic of the present phenomenon and do not occur in mammalian fibrillation. These differences seem sufficient to exclude the identity of the two phenomena. It is, further, generally accepted that mammalian fibrillation is, at least in the auricle, caused by a continuously circulating impulse trapped in the fibrillating tissue (T. Lewis, 1925, and literature there cited), while other workers maintain that it is caused by the activity of a focus or foci of high frequency. Neither mechanism can easily be reconciled with the facts described in this paper, which show that the present form of fibrillation is caused by the telescoping of successive contraction waves and by the creation of impulses in subsidiary pace-makers. The first theory is also rendered inapplicable in the present case by the occurrence of stationary intervals in the fibrillary beat and in some small pieces in cyclic fibrillation; and the second by the absence of any evidence that automatic frequency is increased.*

There is another form of fibrillation occurring in the adult mammalian heart which closely resembles that studied in the present experiments. This is the "micro-fibrillation" of Fredericq (1931), which has also been studied by Delava (1914). It is described as " un fremissement ondulatoire très actif," of very small amplitude, localized in the smallest contractile elements, and much finer than the contractions of macro-fibrillation. It is associated especially with cut surfaces, but can occur in other situations. A profound difference between it and macro-fibrillation is that it can coexist with normal beating; and it also occurs simultaneously with macro-fibrillation. Its presence leaves the normal irritability of the myocardium unimpaired, and it can occur in the smallest pieces of

* *Note added in proof November* 27 (1934)—Evidence obtained since the above was written shows that excess calcium may restore automatic contractility to cells which have become stationary in balanced media.

the heart. In these respects it agrees with the form of fibrillation described in this paper, but one further character points against this identification. Delava was unable to discover it in the hearts of new born cats and dogs, though it occurs in the adults. On the other hand, as stated above, I have observed in embryonic material activity (certainly not caused by potassium, calcium, or magnesium) which agrees very closely both with the description of micro-fibrillation and with the fibrillation caused by these cations. It thus seems probable that the fibrillation described in the embryonic explants in excess calcium, potassium, and magnesium may be more closely related to this micro-fibrillation than to the better known macro-fibrillation of the adult heart.

The question next arises whether the fibrillation produced in the adult heart by excess potassium (Aubert and Dehn, 1874, Wiggers, Thiesen, and Shaw, 1930) or of calcium (Winterberg, 1908), is to be identified with the fibrillation produced in the same material by a different agency (faradization) or with the fibrillation produced in the different (embryonic) material by the same agencies. The circulating wave theory may be regarded as proved, for clinical and faradic auricular fibrillation, at least so far that extremely strong evidence would be needed to displace it, but I am not aware that there is any direct evidence for potassium and calcium fibrillation. The appearance of the two latter resembles that of faradic fibrillation, being a coarser movement than embryonic fibrillation, but this is probably due, at least in part, to the different histological structures of embryonic and adult tissues. On the other hand, Winterberg's description of the fibrillation induced by the injection of calcium chloride closely resembles the appearance of explants in excess calcium, for he found that at a certain stage of intoxication parts of the auricle were in surging " flimmernde " movement, while in other parts co-ordinate beating continued; after further injections the entire auricle passed into fibrillation. This is exactly what I find with explants in different excesses of calcium, except that I find also the occurrence of fibrillation between beats in the beating regions, which Winterberg does not describe. This, however, may easily have been missed if he were not expecting it; or the fibrillary contractions may not have been visible to the naked eye, or may have been suppressed by too high a frequency of beating, so that they would perhaps have appeared if the temperature had been allowed to fall. It appears probable, at any rate, that two kinds of fibrillation exist, of which clinical and faradic fibrillation represent one kind, embryonic cation-produced fibrillation, and probably the micro-fibrillation of Fredericq, the other, while the position of cation-produced adult macro-fibrillation may be regarded as doubtful.

In conclusion, it is a pleasure to express my indebtedness to my wife, to Dr. H. B. Fell and to Dr. A. N. Drury, for much assistance freely given in the course of the work.

Summary

The fibrillation described had the following general characters :—

(*a*) There was lack of co-ordination between adjacent cells. This was, however, not absolute, for neighbouring cells showed a general tendency to contract more or less together.

(*b*) There was often a difference between the form of contraction in the co-ordinate beat and in fibrillation; in the beat each cell always contracted as a whole, but in fibrillation either the same might occur or only part of the cell might contract.

(*c*) Fibrillation might be active throughout the whole heart, or might be confined to almost any large or small area in it.

(*d*) The frequency of fibrillary contractions was higher than that of the co-ordinate beat, and was highest in the sino-atrial region, next highest in the ventricle, lowest in the conus.

(*e*) The amplitude of contraction of the cells was in general shorter in fibrillation than in the co-ordinate beat.

Fibrillation was related to co-ordinate beating in two ways:—

(*a*) In the "fibrillary beat." The co-ordination of the beat was disturbed; it consisted essentially of a number of fibrillary contractions leading up to a more or less perfectly co-ordinate beat originating in the same tissue.

(*b*) In alternate beating and fibrillation. This differed from the fibrillary beat in that true fibrillation occurred between beats, and the co-ordination of the latter appeared perfect; the beat was probably always a response to an impulse originated elsewhere. The same cell might alternately take part in fibrillation and in co-ordinate beats. The occurrence of fibrillation in a beating region depended on the relation between the frequencies of beating and of fibrillary contractions.

Both co-ordinate beating and fibrillation continued when hearts were cut up into fragments invisible to the naked eye. In balanced media, cutting was followed by a transient fibrillation to be identified with the micro-fibrillation of Fredericq; this passed off and was succeeded by normal beating which continued indefinitely. In media which caused fibrillation in entire hearts, similar fibrillation occurred and continued indefinitely in the tiny fragments.

Experiments on the effects of cooling such small pieces, in calcium fibrillation, and then rewarming, led to the following observations (among others): (*a*) Explants originally in fibrillary beat, and the majority of those in fibrillation, displayed when cool regular cyclic changes in activity, periods of maximal activity alternating with periods of minimal activity; (*b*) rewarming explants showing periodicity, and which were originally in pure fibrillation, might cause a transient recovery of some degree of co-ordination.

The following conclusions are drawn as to the mechanism of this form of fibrillation:—

(*a*) The co-ordinate beat suffers dissociation as a result of a reduced rate of travel of the impulse. The time occupied by the passage of each impulse is extended over what should be the stationary interval between beats, with the result that new cycles of activity commence before the old die away.

(*b*) The slow passage of the impulse permits the occurrence of secondary automatic contractions which set up secondary impulses. The result, if the piece be not too small, is the simultaneous existence of a number of entangled impulses.

Similar periodicity could be demonstrated in potassium fibrillation, but it was much less obvious. The mechanism of potassium fibrillation resembles that in calcium, but the impairment of conductivity and the part played by automatic contractions are greater.

In the discussion it is shown that : (*a*) the slow rate of travel of the impulse is probably due to impaired cell to cell transmission, and not to prolonged refractory periods ; (*b*) the present form of fibrillation is distinct in mechanism from mammalian auricular fibrillation, and is probably more closely related to the micro-fibrillation of Fredericq.

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Studies in the Geotropism of the Pteridophyta

V—Some Effects of Temperature on Growth and Geotropism in *Asplenium bulbiferium*

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(*Communicated by W. Stiles, F.R.S.—Received June* 6, 1934)

Although temperature and gravity both influence plant life, and although both factors have been studied for many decades, there is surprisingly little literature dealing with the relation between the two; and none, so far as I can discover, on the effect of either on any Pteridophyte. Navez (1929) who criticized the work of some investigators on the effect of temperature on the geotropism of a few seedlings, sums up the present position in his remark that the conclusions of workers are very different and often in opposition. The present paper gives the results of 1100 experiments carried out mainly between the years 1922 and 1927, and though it is realized that much remains to be done on the question, it is believed that the results which have been obtained are of some value. For general methods, reference may be made to previous "Studies"* in this series. Geotropic sensitivity, as measured by presentation time at different stages in development of the frond, was fully worked out by Waight (1923) for 20° C, and is adopted here as a standard of reference. The growth rate recorded in the tables is that for the particular frond under investigation, or is the average of the fronds examined during the day of the experiment. Nearly all the experiments included in the tables were conducted during the months of April–October, as I have since been able to show that there is an annual rhythm in geotropic irritability. A decrease in sensitivity occurs in winter, and hence experiments performed in November–March are not strictly comparable with those carried out in the summer.

The following abbreviations are used:—

P.S. = period of stimulation.

P.T. = presentation time, *i.e.*, the minimum period of stimulation in a horizontal position, which, under the given conditions, will cause a movement of approximately 5° in about 80% of the fronds.

* For previous studies in this series see literature cited at the end of this paper.

L.T. = latent time (Prankerd, 1925) in hours.

N = "normal time," *i.e.*, the P.T. for different stages of the frond at 20° C (see Waight, 1923).

1—Low Temperature (8° C–20° C)

The work in this section was carried out either in a cupboard with glass doors in the laboratory, or in a cellar where the temperature only altered very slowly. A maximum and minimum thermometer was used, but no thermo-regulator was available. The temperature, however, frequently remained constant within a range of 2° or 3° C throughout the experiment. When it varied to a greater extent, the results could only be employed as a guide, and are not included in the tables. Such methods as opening windows, the use of ice, hot water, etc., were sometimes employed, and it is realized that the conditions were by no means ideal.

Prolonged exposure to low temperatures is accompanied by continued decrease in growth, but after the initial fall, this is slight, and since experiments were performed a few days after exposure to the given temperature, the growth rate was approximately constant for the different series.

Table I gives the details of a few sets of experiments typical of those made at the different temperatures as set forth in the later tables. The stages used of the frond are:—L.1, "late infant" (leaflets still enclosed in the loosening apical coil); A1, A2, etc., "adolescent" with one, two, etc., pairs of leaflets flush with the apical coil. See Waight (1923).

A—8° ± 1° C. This proved a difficult temperature to obtain, and the results in Table II are not conclusive.

The table indicates that the P.T. for this temperature is about 3N, though the number of experiments is scarcely sufficient for its establishment. Moreover, a temperature as low as this could not be obtained for very long, and most of the plants were at a higher temperature (10°–13°) for some days previously. This accounts for the somewhat high growth rate, which after 4 days at 8° C is only about 0·2 cm per day.

B—10° ± 1° C. Table III is a summary of experiments made at this temperature. The plants had been exposed to temperatures approximating to this value for several days before experimentation.

It will be noted that two sets of experiments are given with a P.S. of 2·5N. The first of these was performed in March, and shows a lower sensitivity than that at 2N, thereby illustrating the rhythm before

TABLE I

	Stage of frond	Length of frond in cm	Growth in cm per day	Angle of cuvature °	L.T. in hours
10° C P.S. 2N	L.1	3·0	0·3	5 8	15 17
	L.1	3·3	0 2	5	15
	A4	7·7	0·4	5	15
	A7	7·5	0·4	5	15
	A3	6·9	0·3	—	—
13° C P.S. - 2N	A2	4·95	0·4	20	6·5
	A1	5·7	0·6	12	9·0
	A1–2	4·7	0·6	11	9·0
	A1	5·9	1·2	8	10·0
	A2	8·55	0·9	5	8·0
	A5	7·0	0·6	5	10·0
	A7–8	10·2	0·4	—	—
13° C P.S. - 1·7N	A1	6·9	0·35	5 8	7 12
	A1	7·7	0·3	5	12
	L.1	4·8	0·4	4	12
	L.1	4·5	0·1	3	12
	L.1	4·5	0·4	—	—
16·5° C P.S. - N	A1	6 3	—	3	12
	A5–6	10·5	1·0	—	—
	A1–2	6·5	0·6	—	—
	A1	4·8	0·4	—	—

TABLE II—8° C

P.S.	Average growth in cm per day	No. used	Percentage response	Average angle of curvature °	Average L.T. in hours
6N	—	1	100	10·0	14
4N	0·3	9	67	5·0	20
3N	0·29	10	70	4·7	19
2N	0·4	10	50	5·0	15
1·5N	0·46	4	25	5·0	16

mentioned. The 3N series was also performed in March, and though the sensitivity is the highest in the table, it would probably be higher still if made later in the year. The other 2·5N series indicates that the P.S. is too high, and **2N** seems fairly well established as the P.T. under the

given conditions. This is interesting as it brings gravitational irritability into line with the van't Hoff law—a rise of 10° (of course within a small range of temperature) seems to double the sensitivity.

TABLE III—10° C

P.S.	Average growth in cm per day	No. used	Percentage response	Average angle of curvature °	Average L.T. in hours
3N	0·38	7	86	6·8	13·0
2·5N	0·32	21	71	4·5	13·3
2·5N	0·37	11	82	11·6	13·0
2N*	0·43	36	**78**	5·8	13·1
1·5N	0·35	12	33	6·0	15·0

* The bold type in this and subsequent tables indicates the deduced P.T. and corresponding percentage response.

C—13° ± 1° C. Table IV is a summary of experiments conducted approximately at 13° C, and seems to point clearly to **1·7N** as the P.T. for this temperature. A P.S. of 2N in this case is too high as shown both by the large number responding and the size of the angle of curvature, while the P.S. in the other two series is too low.

TABLE IV—13° C

P.S.	Average growth in cm per day	No. used	Percentage response	Average angle of curvature °	Average L.T. in hours
2N	0·54	16	87	8·3	10·0
1·7N	0·42	36	**78**	5·9	10·7
1·5N	0·46	15	67	5·0	11·5
1·3N	0·48	18	56	6·5	12·0

D—16·5° ± 1° C. Table V indicates 1·3N as the P.T. Although not many experiments were made at this temperature, only one frond out of nine failed to respond to a somewhat higher stimulus, while the value of N at 20°, is based on over 500 experiments. Table VI is a résumé of the work in this section.

TABLE V—16·5° C

P.S.	Average growth in cm per day	No. used	Percentage response	Average angle of curvature °	Average L.T. in hours
2N–1·5N	0·7	9	89	5·0	8
1·3N	0·65	9	**78**	3·6	8
N	0·4	4	25	3·0	12

TABLE VI

Temperature °C	P.S.	Average growth in cm per day	Percentage response	Average angle of curvature °	Average L.T. in hours
8	3N ?	0·2	70	5·0	19
10	2N	0·37	78	5·8	13
13	1·7N	0·48	78	5·0	11
16·5	1·3N	0·65	78	3·6	8
20	N	1·1	80	6·2	5·5

If the results of these experiments are expressed as a graph, fig. 1, where the temperatures are plotted on the x axis and on the y axis the logarithms of the reciprocals of the relative P.Ts., it will be seen that we

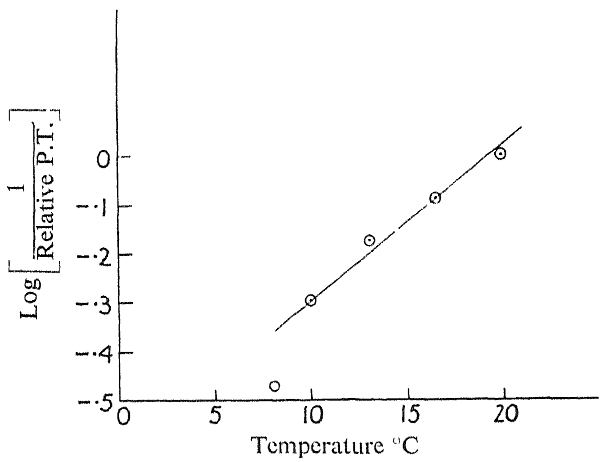

FIG. 1—Relationship between relative presentation times and temperature (P.T. at 20° = 1)

have a good approximation to a straight line. From this it may be deduced that the velocity of the reactions involved increases as the temperature rises according to a formula

$$\text{Log (velocity)} = \text{const.}_1 \times \text{temp.} + \text{const.}_2$$

If the method of calculation due to Arrhenius (1915) be used, it is possible to find a value, μ, for the coefficient of the reaction. The influence of temperature upon a reaction is given by the formula:—

$$K_1 = K_0\, e^{\frac{\mu}{2}\left(\frac{T_1 - T_0}{T_1 T_0}\right)},$$

where

T_1 and T_0 are two temperatures on the absolute scale.
K_1 and K_0 are the velocities of the reactions at the respective temperatures.
e = base of natural logarithms.

For points 2 and 4 on the graph, taking P.T. at 20° C as unity, and computing for absolute temperatures, $\mu = 1 \cdot 096 \times 10^4$, and for points 1 and 3, $1 \cdot 281 \times 10^4$, whence the average value of μ is $1 \cdot 188 \times 10^4$.

A similar graph (fig. 2) constructed from the figures for L.T., also yields a nearly straight line, *i.e.*, the logarithm of the velocity of reaction

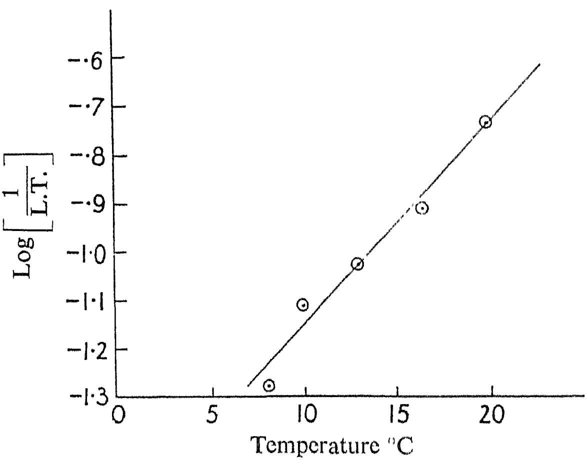

FIG. 2—Relationship between latent time and temperature (L.T. in hours)

varies with the temperature. Evaluating μ as before, we have for points 2 and 4, $1 \cdot 233 \times 10^4$, and for points 1 and 3, $1 \cdot 67 \times 10^4$ or an average value of $1 \cdot 45 \times 10^4$. These results are somewhat lower than $1 \cdot 62 \times 10^4$ which Navez gives for both P.T. and L.T. in the coleoptile of Avena and the root of Vicia. On the other hand, although there are not sufficient points available for accurate evaluations of μ to be made, it is interesting to find how closely these values agree with those quoted by Arrhenius (1915) for biological processes, *e.g.*:—

Assimilation in plants	$1 \cdot 2 \times 10^4$
Respiration in plants	$1 \cdot 48 \times 10^4$
Cell division in eggs (mean value)	$1 \cdot 41 \times 10^4$

In assimilation and respiration it is clear that both physical and chemical reactions are involved, and the values of μ are considerably

lower than in hæmolysis (by acids, bases, and lysins), where the values run from $2 \cdot 5 \times 10^4$ to 3×10^4, and where the small dimensions ensure that the physical process of diffusion is practically negligible and chemical change is paramount. Hence the results point to the likelihood that both chemical and physical changes are involved in the processes of geotropic reaction.

2—High Temperature—(20°–35° C)

Waight gave 1·4 cm per day as a constant growth-rate for adolescent stages at 20° C. This can, however, be regarded as only approximate, since the rate varies with several factors notably the size of the frond. A rough approximation for daily increments of growth in the greenhouse where all experiments at 20° C were made (Prankerd, 1925) is 1·0–1·1 cm. Since constant temperatures above 20° C could not be obtained in the greenhouse, incubators were used which were kept constant within a degree of the desired temperature. They had glass doors and were lined with white paper, so that the conditions should be as far as possible similar to those of the greenhouse. Fig. 3 shows the graphs for daily increments of growth at 20° (greenhouse), 20°, 25° and 30° (incubators). The 20° incubator was used as a control in order to test how far the conditions approximated to those of the greenhouse, and the fact that the growth-rate declined showed that they were not identical. Nevertheless that no condition was inimical to geotropism to any extent was shown in that experiments performed on plants grown in the 20° incubator—even after four days—gave similar results to those made in the greenhouse. Hence it may fairly be inferred that the results obtained from plants grown for a few days in an incubator at another temperature were due to the effects of that temperature.

The initial rise of the growth-rate at 25° and 30° is very striking, and due no doubt chiefly to the effect of shock (Lundegårdh, 1931). In the subsequent decline of growth activity curious alternating daily periods are noticeable. These are seen to some extent, though fitfully and irregularly, at the constant temperature of the greenhouse, and in this case must be due to internal causes. The sudden change of temperature clearly acts as a stimulus to growth which cannot be maintained, and it is perhaps this initial rise and fall which accentuates the tendency to alternating periods of growth whose significance is obscure.

On one fortunate occasion, it was found possible to carry out a series of experiments in the greenhouse where the temperature remained constant throughout at 25°. Two other series were performed in an incubator

at 25° after 1–4 days' exposure. In all experiments a P.S. of 0·8N was used, and the results are set out in Table VII.

The L.T. is always much increased in the winter (see p. 479), but the work in the summer months points to a value slightly less than 0·8N as the P.T. for 25° C.

The initial effect of a temperature of 30° C on growth is markedly opposite from that on graviperception. The growth-rate rises on the

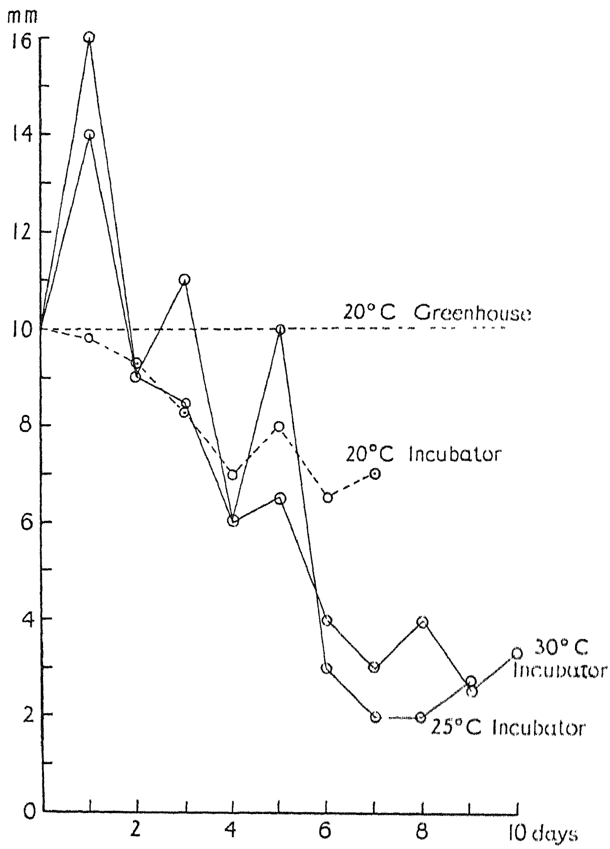

FIG. 3—Average daily increments of growth in fronds of *Asplenium bulbiferum*

TABLE VII

Condition	Month	Average growth in cm per day	No. used	Percentage response	Average angle of curvature °	Average L.T. in hours
Greenhouse	June	1·1	9	90	9	4·0
Incubator	July	1·5	5	80	10	5·4
,,	November	0·7	5	80	5	8·0

first day, though not to so great an extent as at 25°, while perceptive power suffers a considerable decrease. After the first day the effect of this temperature is inimical to both processes, though to what extent could not be determined under the given conditions. Table VIII gives the results of experiments on plants at 30° after 1 hour's previous exposure. This was done so that the fronds should be at 30° for the whole period of stimulation.

TABLE VIII

P.S.	No. used	Percentage response	Average angle of curvature °	Average L.T. in hours
1·7N	3	100	10·0	5·7
1·33N	18	**78**	5·7	5·25
1·25N	12	42	6·0	5·5
N	10	30	5·0	5·25
N−	2	0	—	—

That the stimulus must be increased by one-third to produce 80% response is a clear deduction from the figures. It is equally clear that the L.T. is scarcely affected since it is practically 5½ hours which is normal for 20° (Waight, 1923).

TABLE IX

P.S.	No. used	Percentage response	Average angle of curvature °	Average L.T. in hours
2·5N	1	100	15·0	5·0
2N	2	100	9·0	5·0
1·7N	11	**82**	5·0	6·2
1·5N	28	54	5·7	5·5
1·33N	17	41	4·1	5·5
N	2	0	—	—
N−	2	0	—	—

Table IX gives the results of experiments on plants at 30° after one day's previous exposure. Here the P.T. has to be increased by two-thirds, showing that 30° is a progressively injurious temperature for graviperception in this plant, though the rate of reaction as measured by the L.T. is still unaffected. Experiments made after more than a day's exposure, though insufficient to establish relative irritability, indicate (1) that the power of graviperception and response continues over a week at least, and (2) that the L.T. has a constant approximate value of 5½ hours. It may here be remarked that at this temperature the stage of

the frond, *i.e.*, late infant and adolescent to A8, does not affect the L.T. At 35° there is little if any reaction to gravity, and fronds tend to wilt after several days' exposure.

Leitch (1916) has drawn a clear theoretical distinction between the optimum temperature for a vital process (*i.e.*, that at which the maximum constant rate occurs), and the maximum rate temperature where a time factor is involved. But the two points may be very close to one another, as in her own experiments (28°–30° and 30·3° C respectively for growth in the roots of Pisum sativum). Neither value could be determined exactly for growth or geotropic reaction in *Asplenium bulbiferum*, though the work indicates that these points must be very near 25° for both processes.

Comparing the effect of high and low temperatures upon gravitational irritability, we see that travelling down the scale from the optimum, the P.T. and L.T. are equally affected; while passing upwards, a time factor operates,* the P.T. increasing progressively, though the L.T. after an initial rise remains constant.

3—Varying Temperatures

This section deals with a part of the work in which the plants were exposed to two different temperatures during the experiment, so arranged that the response took place at a temperature other than that at which the stimulus was given. In the winter of 1921–22 my student, Miss Waight took advantage of a thick layer of snow to stimulate some fronds at 0° C. She found that scarcely any growth and no reaction to gravity took place, even after four or five days; but when the plants were removed to 20° C unmistakable response occurred. Czapek (1895) showed that roots of lupin stimulated at 2° C will not respond at that temperature, but will do so at 19° C. He, however, made no attempt to discover the P.T., and records no growth measurements. The effect of various periods of exposure to a temperature of 0° C seemed worth quantitative investigation, but since continued snow was unfortunately not available, a tank with an ice jacket was used in the cellar in which the low temperature experiments described in Section I was made. The ferns were left for a few hours in the cool cellar (8°–15° C) in order to avoid the effect of shock from so steep a gradient as 20°–0°. They were then placed upright in the tank for about an hour to prevent any effect resulting from the higher temperature before the plants had cooled down. Fronds were

* It no doubt does so with low temperatures, but so slowly as to be negligible within a considerable period of time.

always measured before and after placing in the tank, but the difference was usually nil. In some fronds growth from 0·05–0·2 or even 0·3 cm had taken place; in a few slight decrease in length was observed, while, a very few wilted. Occasionally a slight movement, apparently geotropic had occurred. After stimulation the plants were replaced in the green-house at 20°, any movements noted, and the fronds measured each day, for the three, or sometimes four, following days. The results for some representative series of experiments are given in Table X. For exposures lasting more than a day, the stage of the frond was not taken into account, and the plants were all removed from the tank at the same time. But when the periods of stimulation were measured in hours only they were made to vary with the stages of the fronds employed, and are therefore expressed, like those in previous tables, as multiples of the P.T. at 20° C. The fourth column of Table X gives the average period elapsing between the end of stimulation and the beginning of movement, *i.e.*, L.T.–P.T. This period was termed "transmission time" by Tröndle (1910), not perhaps very happily, since it implies that perception and transmission are separate entities taking place in different periods, while it would seem more probable that they are to a consderable extent synchronous. Table XI is a summary of experiments 0° → 20°.

The average growth per frond for all experiments on the first four days is respectively 0·49, 0·75, 0·99 and 1·0 cm, which shows that the nomal rate is attained after 2 days at 20° C irrespective of the duration of the previous exposure to 20° C. It is evident from the table that a P.T. as here defined (p. 479) does not exist; for however much the stimulus is increased, the response falls considerably below 80% (if sufficient numbers are used), while the average angle of curvature (11·3°) is far above normal. In other words the relationship of the numbers responding to the amplitude of response discovered for 20° C does not hold after exposure to a temperature of 0° C. There is need for further investigation, particularly as the work at this extreme temperature supports Paal's finding (1913) that individual variation increases with departure from optimum conditions (*cf.* p. 479).

Prolonged exposure to a temperature of 0° C does not appear to affect the vitality of the plants. The growth-rate for the first day at 20° (average 0·5 cm) is the same whether the plants are previously exposed to 0° for a day or a fortnight, and the time of recovery (L.T.–P.T., *i.e.*, 2 days), is remarkably constant after exposure from 2 days upwards. The figures for percentage response and angle of curvature are not so steady, but there is no progressive increase with the time of exposure from 6N upwards.

TABLE X

P.S.	Stage of frond	Length of frond in cm	Average angle of curvature	L.T.-P.T.	Growth in cm 1st day	2nd day	3rd day	4th day
			°	days				
7 days	L.1	4·9	20	2	0·25	0·35	0·9	0·9
	A.1	5·0	10	2·5	0·4	0·35	1·3	
	A.4	7·1	5	4·0	0·4	0·45	0·35	0·6
	L.1	3·9	5	2·0	0·55	0·66	0·9	1·1
	L.1	4·3	3	1·5	0·5	0·4	0·5	1·0
	A.5	13·8	—	—	1·2	0·8	0·7	1·0
	A.2	8·5	—	—	1·0	0·85	0·95	1·0
	A.2	7·3	—	—	0·55	0·7	0·4	0·8
	L.1	5·5	—	—	0·55	0·7	0·65	0·95
	L.1	3·1	—	—	0·3	0·4	0·5	0·7
3 days	A.6	13·1	20	1·25	0·2	0·3	0·6	—
	L.1	3·0	20	2·0	0·6	0·1	0·3	—
	L.1	4·2	15	2·0	0·1	0·4	0·5	—
	A.1	7·9	10	*	0·6	0·8	1·8	—
	A.1	3·8	10	1·25	0·4	0·4	0·4	—
	L.1	3·7	5	2·25	0·0	0·1	1·1	—
	A.1	7·5	2	2·5	0·5	1·0	1·1	—
	A.7	14·1	—	—	0·9	0·5	0·5	—
	A.7	10·75	—	—	0·75	0·5	1·0	—
	A.6	13·6	Wilted	—	0·5	0·5	1·6	—
	L.1	4·2	—	—	0·2	0·2	0·8	—
				hours				
6N	A.7	13·6	20	12·5	0·5			
	A.6	19·0	5	12·0	1·0			
	A.5	9·2	5	13·0	0·7			
	A.4–5	12·0	5	9·0	1·2			
	A.6	15·0	3	9·5	0·7			
	A.6	11·5	3	13·0	0·9			
	A.5	11·0	—	—	0·9			
	A.3–4	7·9	—	—	0·4			
	A.3	12·5	—	—	0·6			

* The movement of 10° took place in the tank, and the L.T. is therefore unknown.

Further sets of experiments were made in order to determine the differential effect of 30° and 20° on stimulus and response. In the first set the fronds were stimulated in the usual way at 20°, and after 2–3 hours (*i.e.*, about half the L.T.), the pots were transferred to the incubator at 30°, where response, if any, took place. A second set of plants was placed upright in the incubator for periods varying from 20 minutes to

TABLE XI

P.S.	No. used	Percentage response	Average angle of curvature °	L.T.–P.T. (average)	Average growth in cm 1st day	2nd day	3rd day	4th day
				days				
13 days	8	63	8·0	2·0	0·4	1·0	—	—
10 days	14	79	12·9	2·1	0·5	0·8	1·3	1·2
7 days	19	58	8·9	2·1	0·6	0·6	1·0	0·9
5 days	10	40	6·3	1·7	0·5	0·7	0·9	—
3 days	13	69	11·1	1·9	0·5	0·4	0·9	—
2 days	16	31	10·0	1·2	0·5	0·7	0·8	—
				hours				
1 day	13	62	18·1	10·8	0·4	1·0	0·9	—
12N	6	83	14·0	8·3	0·9	1·5	1·4	—
9N	8	50	11·3	8·2	—	—	—	—
6N	22	68	6·8	10·4	0·6	—	—	—
3N	11	45	9·6	10·7	0·6	—	—	—
2N	2	0	—	—	0·9	—	—	—

2 hours, then stimulated, and again placed upright. After about 3 hours in all they were removed to the greenhouse. No difference was noticed corresponding to the time, early or late during the period of exposure to 30° when the stimulus was given, which was N in both series. The results of these experiments are summarized in Table XII.

TABLE XII

	No. used	Percentage response	Average growth in cm during experiment	Average angle of curvature °	Average L.T. in hours
20° → 30°	17	82	0·23	7·3	5·0
30° → 20°	17	35	0·29	4·8	5·5

In the first set it will be seen that the effect of the high temperature on the response is scarcely appreciable. If anything it is mildly beneficial, as the movement is a little greater and the L.T. slightly less than the corresponding quantities when the response takes place at 20°. In the second set, while the angle is not greatly affected, we have the striking result of a fall in the number responding of 56%. The L.T. is not affected, as was shown in the preceding section for longer exposures. Every effort was made to use fronds as similar as possible in each set of experiments, but the growth-rate is somewhat higher in the second set of

plants. This is of interest as showing that the feeble response was not due to inferiority in the fronds, and the first set of experiments as well as those recorded in Section 2, show that it is not due to interference in the mechanism of response. It seems clear therefore that a high temperature immediately injures those early processes in the chain of geotropic reaction which we conveniently term perception.

The mathematical treatment of the results for low temperatures was carried out by my friend Miss M. Williams, F.L.S.; and my warm thanks are given to her not only for the time and trouble she expended, but for her sympathetic interest throughout the work. I also wish to express my appreciation to Professor Stiles, F.R.S., for reading this manuscript; and to my students Miss Waight and Miss Brain, F.L.S., for their ready and valued help in some parts of the experimental work.

Summary

1 *Low Temperatures*—The growth-rate, presentation time, and latent time at 8°, 10°, 13°, 16·5° and 20° are recorded. At 10° the presentation time is double that at 20°, which points to chemical changes acting in accordance with the van't Hoff law. The growth-rate is about one-third, and the latent time a little more than double that at 20°.

The coefficient of reaction as calculated from these results is $1 \cdot 188 \times 10^4$ and $1 \cdot 45 \times 10^4$ for presentation time and latent time respectively, and these values are comparable with those which have been deduced for some other physiological processes.

2 *High Temperatures*—The growth-rate, presentation time, and latent time at 30° are recorded, and a time factor is shown to operate. This temperature is progressively injurious to the power of graviperception at once increasing the presentation time by one-third, and by two-thirds after one day's exposure. The latent time is the same as that at 20°, and is not affected by the time factor. 25° is probably near the optimum temperature for growth and graviperception in this plant.

3 *Varying Temperatures*—(1) 0° → 20°. Experiments are described where the stimulus was given at 0°, and reaction took place at 20°. The angle of curvature is high, though 80% response is unobtainable. Prolonged exposure to a temperature of 0° C does not affect vitality as shown by subsequent growth-rate, number responding, amplitude of response, and latent time.

(2) 20° → 30°. These experiments show that the injurious high temperature affects the initial (" perception ") and not the end ("response") links in the chain of geotropic reaction.

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Investigations on Mediterranean Kala Azar
VII—Further Observations on Canine Visceral Leishmaniasis

By S. ADLER and O. THEODOR (Kala Azar Commission of the Royal Society)

[PLATES 23 AND 24]

(*Communicated by Sir Henry Dale, Sec. R.S.—Received June* 18, 1934)

In a previous communication observations on canine visceral Leishmaniasis made in Malta from May to the end of October, 1931, were recorded. Special attention was paid to the skin which was found to be uniformly affected and its importance as a source of infection for sandflies was stressed. From May to the end of November, 1932, 188 more dogs were examined by the method previously described and 19 (*i.e.*, circ. 10%) were found infected. It would therefore appear that the conditions with regard to canine visceral Leishmaniasis in Malta have remained fairly uniform over a long period, for Wenyon (1914) found 6 out of 46 (*i.e.*, 14%) and Critien (1911) 10 out of 83 (*i.e.*, 12%) of dogs infected by examination of spleen smears.

On the basis of the above figures there are during the summer months at least 1500 dogs in Malta capable of infecting sandflies (Professor V. Bernard, of the Department of Health, Malta, estimates the number of dogs on the island at about 15,000). Actually the above figures are an underestimate, for as will be shown later parasites in the spleen of an infected animal may be so few as to be overlooked even after a prolonged examination of smears, although sandflies fed on the same animal become infected. In order to determine the infection rate approximately it would be necessary to examine animals by cultures of the spleen juice and bone marrow or, what is hardly practical on a large scale, to feed sandflies *P. perniciosus* or *P. major* on the animals and to examine the insects for flagellates. Histological examination of the skin would fail to detect the infection in some animals on which sandflies *P. perniciosus* infect themselves to the extent of 20%.

A review of the literature indicated that the disease is seasonal and it was therefore concluded that some of the infected animals die or

become cured spontaneously during the winter months. This appears to occur in Malta, for Dr. A. Critien, Director of Health for Malta, informs us that between December, 1932, and March, 1933, two hundred animals were examined and only eight were found infected. This conclusion only applies to animals with a visceral infection sufficiently intense to be detected by examination of spleen smears and the proportion of infected animals which escape detection by this method is not yet known. From the epidemiologist's point of view the latter group of animals are important for they serve as a reservoir for infecting sandflies.

Fully 60% of the infected animals appeared to be well nourished and in good health. As previously found the commonest sign observed was seborrhea with patches of depilation appearing firstly near the eyes; other signs noted were emaciation, conjunctivitis, and opacity of the cornea. Opacity of the cornea in one or both eyes is not uncommon and may rapidly lead to almost complete blindness. In one case necrosis of the lower jaw and surrounding tissues was found in a heavily infected and moribund animal.

In some animals the intensity of the infection bears no relation either to the clinical condition or to the histological findings; an animal showing numerous parasites in the spleen may be in good health, while another, in which the infection is so slight that it can only be demonstrated by culture of spleen juice or sandfly feeding experiments, may be moribund.

Naturally infected animals have been observed for a period of six months before exitus. In experimentally infected animals the disease may run an acute course and end fatally in five months in spite of a slight infection. External signs of infection (seborrhea and keratitis) may appear as early as two to three months after inoculation.

Pathology of Canine Visceral Leishmaniasis

The pathology of canine visceral Leishmaniasis has been described by various authors and more recently by Redaelli (1933) on the basis of a detailed histological study of six animals from our series. Redaelli gives a practically complete bibliography. It was intended in this paper to supplement current accounts of the pathological findings in dogs, and as far as possible to attempt to elucidate the frequent discrepancies between the clinical conditions and the intensity of the infection. This was not quite possible on the basis of findings in naturally infected and uncontrolled animals, and it was therefore decided to supplement these findings by a study of experimental infections. A total of nineteen animals were used for histological study; of these six died from natural

infections, two from experimental infections, and the remainder (naturally infected) were sacrificed.

The following is the history of the two experimental animals:—

Dog No. 1—Inoculated intrahepatically September 23, 1932, with infected bone marrow from the tibia of a child in Professor A. Longo's clinic in Catania. The bone marrow was kept on ice for two days in a thermos flask and transported to Malta where the inoculation was performed. Spleen smears taken from the animal several weeks before the inoculation were negative. Although it was subsequently found that negative spleen smears do not necessarily prove freedom from infection, it is probable that in this case the disease which developed in the dog was actually due to the inoculation, because the histological findings in the liver were different from those of naturally infected animals.

At the end of November, 1932, the animal appeared in excellent condition and was transported to Jerusalem where it was kept under observation. Towards the middle of February, 1933, seborrhea and depilation were observed. On April 5 and 9, 1933, 31 laboratory bred *P. perniciosus* were fed on the animal and 10 were subsequently found positive. The animal became progressively emaciated and died on April 14. The spleen was not enlarged, spleen smears were negative, but the spleen juice gave cultures. A few L.D. bodies were found in smears of bone marrow.

Dog No. 2—Spleen pulp from Dog No. 1 was inoculated intrahepatically into Dog No. 2 (April 14, 1933). By the middle of July, running at the eyes, depilation round the eye-lids, and opacity of the cornea were noted. The opacity of the cornea developed very rapidly, and in the first week of August the animal could only distinguish between light and dark. In spite of a moderate appetite the animal became progressively emaciated and died on September 26, 1933.

The spleen was not enlarged, but spleen smears showed a few parasites. Two cats inoculated intrahepatically at the same time as Dog No. 2 were examined on November 21, 1933, and found negative.

These two dogs were therefore typical instances of slight but nevertheless fatal infections without any other complications such as pneumonia to which infected dogs and children are particularly susceptible. All the naturally infected animals in our series, many of which were apparently in good condition, contained more parasites in the spleen than the above two animals. Histological examination of the liver and bone marrow in these two cases also revealed relatively few parasites, and it was therefore

clear that the investigation of the liver, spleen, and bone marrow on which the attention of previous investigators had been focussed could not elucidate the fundamental pathology of the disease. It appeared that the only logical method of attacking the problem was a detailed study of tissues of naturally and experimentally infected animals with the object of determining the factors common to all fatal infections, irrespective of their intensity.

Skin—The histological changes in the skin have already been noted, and in their main outline have been confirmed by Redaelli. It has already been pointed out that the infection in the skin may be more intense than that of the spleen, but this does not necessarily indicate that the skin is the seat of primary infection, for a considerable skin infection and a very slight infection in the spleen may occur in animals inoculated intrahepatically.

As was to be expected, the infection rate in sandflies increases progressively as the infection of the skin becomes more intense, till finally in some infected animals an infection rate of almost 100% is produced in *P. perniciosus*. The following examples will suffice:—

	Dog No. 3			Dog No. 8			
	No. fed	No. pos.	%	No. fed	No. pos.	%	
May-June..........	52	8	15·4	72	0	0	June.
July	29	9	31	13	3	23	July-August.
October	39	30	79	45	32	71	Sept.-October.

The infection of the skin even when moderate appears to be uniform. Thus between August 21 and August 27, 1932, Dog No. 108 produced an infection rate of only 30% in *P. perniciosus* and the histological examination of the skin showed a relatively slight infection. Sandflies *P. major*, which are even better hosts for *L. infantum* than *P. perniciosus*, allowed to feed at random at the same time on the same animal over an area of skin of about six inches square gave an infection rate of 80%, *i.e.*, at least one infected macrophage occurred in every 1000 sq. μ of the skin.

Parrot, Donatien and Lestocquard (1933) attach no particular importance to the infection of apparently normal skin. We cannot agree with this opinion, for the infection includes the nose, inner part of the external ear, and the less hairy parts of the abdomen, sites on which sandflies feed readily. It must also be born in mind that *P. perniciosus* feeds much more readily on dogs than on man, both under laboratory conditions

and in nature. In cases where there is depilation the possibilities for the feeding of sandflies are further increased. The infection of the unbroken skin is the deciding factor in the propagation of the flagellates in sandflies. Infected skin which is ulcerated and contaminated by secondary infections is not of the same importance, for sandflies which ingest bacteria die within a few days.

Eye and appendages—Donatien and Lestocquard rightly attach importance to the signs in the eyes. Running from the eyes and loss of the eyelashes are common. This is due to infiltrations of macrophages and plasma cells in varying proportions in the eyelids, both on the external and on the conjunctival surfaces and more particularly round the Meibomian glands.

In animals which show no external changes in the eyes, the corneo-sclerotic junction is frequently the site of an infiltration consisting either entirely of macrophages (figs. 1 and 2, Plate 23) or macrophages and plasma cells and, as in other sites, the extent of the infiltration may bear no relationship to the number of parasitized macrophages. Both infected and non-infected macrophages frequently ingest melanin.

Infected macrophages frequently wander from the corneo-sclerotic junction into the substantia propria of the cornea, sometimes without causing the slightest local reaction. They can usually be found in the anterior part of the cornea near Bowman's membrane and very rarely near Descemet's membrane. Occasionally infected cells which have wandered into the cornea disintegrate and leave a deposit of extracellular L.D. bodies.

Five animals in our series showed opacity of the cornea. In such cases there is a slight vascularization at the periphery of the cornea together with a dense infiltration of various proportions of macrophages and plasma cells in the anterior part of the cornea. In one experimental animal the infiltration in one part of the cornea was sufficiently dense to interfere with nutrition of the epithelium and initiate ulceration (figs. 3 and 4, Plate 23).

The wandering habits of the macrophages are clearly shown in the cornea where individual cells stray a considerable distance from the infiltrating mass in the corneo-sclerotic junction towards the centre of a normal cornea. These cells occasionally pass through Bowman's membrane and force their way between the epithelial cells.

Infection of the eyelids is always found while the other changes are inconstant, the most frequent being infiltration at the corneo-sclerotic junction.

Liver—The changes in the liver are similar to those previously described in experimental hamsters, but are less intense. There is an important difference between the histological changes in naturally infected animals and those inoculated intrahepatically. In the former there is always a considerable infiltration of macrophages and plasma cells round the interlobular veins in addition to infection and proliferation of the Kupfer cells; in slight infections of the latter group, changes in the liver occur in the Kupfer cells which may proliferate sufficiently to obliterate the lumen of capillaries and there is in addition infiltration round the intralobular veins (fig. 7, Plate 24). In the two experimental dogs described above in which the infection of the spleen was very slight there was hardly any change round the interlobular veins, but there were numerous infiltrations round the intralobular veins. These observations clearly indicate that in naturally infected animals the changes round the interlobular veins are mainly due to the invasion of infected macrophages via the branches of the portal veins from the spleen and intestines. There can be no doubt on this point, for in experimental animals such as hamsters and spermophils large numbers of infected macrophages are always found in the portal circulation where they are more numerous than in the systemic veins. Redaelli (1933) who had no opportunity of examining experimentally infected dogs thought that the specific infiltration round the interlobular veins might be due in the first instance to invasion of the virus through the general circulation at the time of infection and not secondary to the infection of parts drained by the portal veins.

A slight or moderate infection of the liver acts as a powerful stimulus to phagocytosis and the Kupfer cells are frequently found ingesting polymorphs, lymphocytes, and red cells indiscriminately.

Spleen—As previously noted, the spleen may be normal in size, enlarged, or small and cirrhotic. The only constant findings in the spleen are large numbers of plasma cells which are specific for canine visceral Leishmaniasis, and fibrosis which is probably an immunological reaction since it is not seen in experimental animals such as hamsters and spermophils which never show a tendency to spontaneous cure. In animals with a chronic infection thick diffuse white patches are found in the capsule and these on section are found to contain numerous plasma cells and infected macrophages (fig. 5, Plate 24).

Bone marrow—Infected macrophages are always found in the bone marrow even when there are very few in the spleen. The megakaryocytes

are often increased in number, sometimes to a very striking degree (fig. 6, Plate 24); but this change is not constant.

The heart—Redaelli has observed foci of infection in the heart muscle. The animals in which the disease ended fatally all showed degenerative changes in the heart muscle and in addition congestion in the capillaries of the liver secondary to heart failure.

Voluntary muscle—In one animal with a heavy natural infection which ended fatally small foci of infiltration with numerous plasma cells and a few macrophages were found scattered among the fibres of voluntary muscle.

Adrenals—Infected macrophages are constantly found in the cortex and at the junction of the cortex and medulla. The cortex is always enlarged. The infection in the adrenals differs from that of other organs in the absence of secondary infiltration of plasma cells.

Bladder—In heavy infections the bladder always contains numerous infected macrophages lying scattered among the muscle fibres, either in discrete masses, or as individual cells under the epithelium. The amount of plasma cell infiltration is variable. The condition may be described as one of specific chronic cystitis.

Stomach and intestines—We consider that the changes in the digestive tract are the most important of all for the elucidation of the clinical pathology of canine visceral Leishmaniasis for *they are the only ones common to all fatal infections*. They consist of infiltrations of plasma cells and macrophages in the mucosa and submucosa. In no case did we find ulceration. The infiltration may be intense even in slight local infections and in fatal infections the plasma cells in the mucosa and submucosa of the intestines may almost entirely replace the lymphocytes. It seems that this intense infiltration interferes with absorption of food, although the epithelium of the mucosa is intact. We can find no other explanation for the emaciation present in all the fatal infections, two of which were so slight that they might easily have been overlooked in examinations of spleen smears.

Kidneys—In heavy infections the kidneys are always involved and may contain numerous parasites. The following changes were noted:—(*a*) infiltration of plasma cells round the arterioles; (*b*) scattered foci of macrophages and plasma cells independent of the arterioles; (*c*) individual infected macrophages in contact with Bowman's capsule and the tubules. Infected macrophages sometimes work their way into the interior of the tubule so that their elimination in the urine is inevitable. These

findings are not constant and in two fatal infections no marked changes were found in the kidney.

Thyroid--Infected macrophages were found in two heavily infected animals, but without any obvious histological changes.

Other tissues--Infected macrophages were found in the urethra, vagina, nasal mucosa, pharynx, tongue, and mucosa of the mouth. In Dog No. 2 the infection in the mucosa of the mouth was heavier than that of any other site. In view of the migratory habits of infected cells and their capacity for passing through soft tissues (*e.g.*, the conjunctiva) the discharge of infected cells in excretions (from the mouth and nasal discharges or urine) of heavily infected animals appears to be inevitable. Nevertheless, infections through the above sources cannot be considered to be of any importance. An infection capable of spreading in dogs by this method would soon become universal, but in Mediterranean countries the disease is limited in its distribution to that of sandflies of the *major* group.

The L.D. bodies from the spleen smears of naturally infected dogs are generally larger than those found in the spleen and bone marrow of infected children, but this is of no particular significance as L.D. bodies vary in size in different hosts, *e.g.*, *L. infantum* of human origin inoculated into *Cricetus auratus* attains the same size as in naturally infected dogs while *L. tropica* of human origin inoculated into mice may produce L.D. bodies three or four times the size of those found in human lesions.

The circulating blood both in man and dogs is not a suitable medium for infected cells, and it is unusual to find infected macrophages in blood smears of even heavily infected cases, although they are present in small numbers and can be demonstrated by blood culture in 91·7% of infected children.

Infected macrophages are found in all tissues except the central nervous system, ovary, and testes. During the last four years we have carried out histological examinations of numerous animals (*Microtus güntheri*, *Citillus citillus*, *Cricetus auratus*, *Cricetus griseus* and dogs) without finding a single instance of infected macrophages in the brain, although in spermophils infected cells are found in the choroid plexus.

The tissue reactions and intensity of infection vary not only in different animals but in different organs of the same animal; a moderate infection of the skin may be accompanied by a hardly recognizable infection in the spleen, an animal with a heavy infection may show no change in the cornea while another with a slight visceral infection may be blind

through keratitis. The secondary reaction is also variable; in some cases the presence of a few infected macrophages induces an intense proliferation of non-infected macrophages particularly round hair follicles, sebaceous glands, and at the corneo-sclerotic junction, while in others the proliferation is slight. The plasma cell infiltration which accompanies that of the macrophages is also variable and may be out of all proportion to the infection; it may occur in any infected tissue, but is constant in the spleen, lymph glands, bone marrow, and intestines.

The spread of the infection appears to be as follows: Infected macrophages are carried by the blood stream and deposited in various tissues. These infected cells are active and capable of phagocytosis as evidenced by their ingestion of melanin in the skin and corneo-sclerotic junction and lymphocytes and polymorphs in other tissues, but they are incapable of dividing and have never yet been found in mitosis, though they are capable of inducing proliferation of non-infected macrophages and plasma cells in their immediate neighbourhood. On the death of a parasitized cell the L.D. bodies are liberated and ingested by neighbouring macrophages, and in addition the infection may extend locally by interchange of cell contents between neighbouring cells.

Pittaluga (1927) summarized the pathology of visceral Leishmaniasis as a blocking of the R.E. system and other authors, Adler (1929) and Redaelli (1933), have pointed out that the L.D. body is a parasite of the R.E. system. It is not justifiable to consider visceral Leishmaniasis as a blocking of the R.E. system, for many of the macrophages even in heavy infections are capable of phagocytosis and the Kupfer cells ingest polymorphs and lymphocytes, and both infected and non-infected macrophages often phagocytise melanin granules in the skin and at the corneo-sclerotic junction. In this connection it is interesting to note that children suffering from infantile Kala Azar are practically immune to malaria, and Paradiso working in Catania found only one case of malaria among two thousand infected children, of which the majority came from endemic centres of malaria. Blocking of the R.E. system would be expected to produce susceptibility to malaria which takes a more severe course in splenectomized than in normal monkeys. Actually infection with infantile Kala Azar appears to give some degree of protection against the diseases of childhood, for Zahra (1933) has shown that the mortality among infected children treated mainly as out-patients is smaller than in other children of the same age-group.

Although the histological findings in canine visceral Leishmaniasis are similar in some respects to those of infantile Kala Azar there are impor-

tant differences between the pathology of the human and canine disease. In the former infection of the skin and keratitis have never been found, while the spleen is constantly enlarged. Infection in children is always accompanied by severe symptoms and a heavy infection in the absence of clinical signs such as is found in dogs is inconceivable. In children proliferation of macrophages in the spleen, even when parasites are so few as to be overlooked in smears of the spleen and bone marrow as is the case in some relapses, suffices to produce the typical symptomatology of Kala Azar. There is not sufficient pathological material for comparing the intestinal changes of infected dogs with those of children, but it is significant that when diarrhœa indicating changes in the intestine occurs in infantile Kala Azar the disease is often fatal in spite of intensive treatment.

The results of feeding experiments with sandflies in naturally infected dogs will be dealt with in a further communication.

We have to thank Dr. A. Critien, Director of Health, Malta, and Professor V. Bernard for their kindness in providing us with laboratory accommodation and facilitating our work in Malta in every possible way.

We have to thank Professor P. Redaelli, of the University of Catania, for placing his laboratory and equipment at our disposal.

Summary

The pathology of canine visceral Leishmaniasis is described. Infected animals may be apparently healthy. Infection of unbroken skin is present in all naturally infected animals, and in animals inoculated intrahepatically. The end result of the infection does not depend on its intensity. Infection of the eyes, mouth, nasal mucosa, and urinary passages may be such as to render the escape of parasites through discharges inevitable, but this is of no importance for the spread of the disease.

The infection of the spleen may be so slight as to be overlooked in spleen smears.

The histological findings in animals which succumb to the disease vary. The only factor common to fatal infections is infiltration of the intestinal mucosa and submucosa with macrophages and particularly with plasma cells.

It is considered that the intestinal changes are responsible for the emaciation which precedes death in fatal uncomplicated cases.

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Explanation of Plates

Plate 23

Fig. 1—Patch of infiltration at corneo-sclerotic junction. (Remainder of cornea was normal.) From naturally infected dog. × 80.

Fig. 2—Massive infiltration at corneo-sclerotic junction in dog infected by intra-hepatic inoculation. × 80.

Fig. 3—Diffuse keratitis. Infiltration throughout substance of cornea, most marked in anterior part. Thinning of epithelium at one point (pre-ulceration). × 80.

Fig. 4—Part of fig. 3 (indicated by dotted line) more highly magnified, showing pre-ulceration. Massive infiltration of macrophages with very few parasites. × 320.

Plate 24

Fig. 5—Thickening and infiltration in capsule of spleen from naturally infected dog. × 80.

Fig. 6—Bone marrow showing increase of megakaryocytes. From experimentally infected dog. × 120.

Fig. 7—Congestion of liver and infiltration round intralobular vein in experimental infection. × 120.

Fig. 8—Plasma cell infiltration between the villi of the small intestine in a naturally infected dog which died of inanition. × 560.

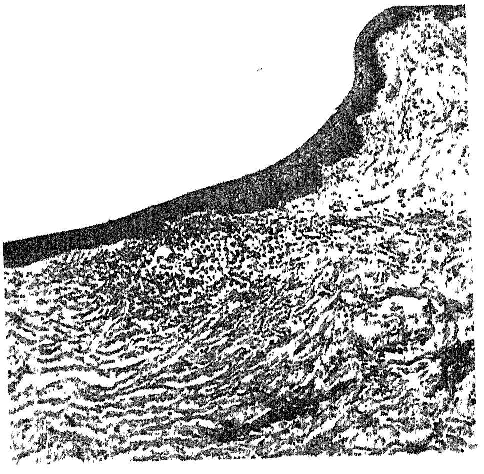

FIG. 1

FIG. 2

FIG. 3

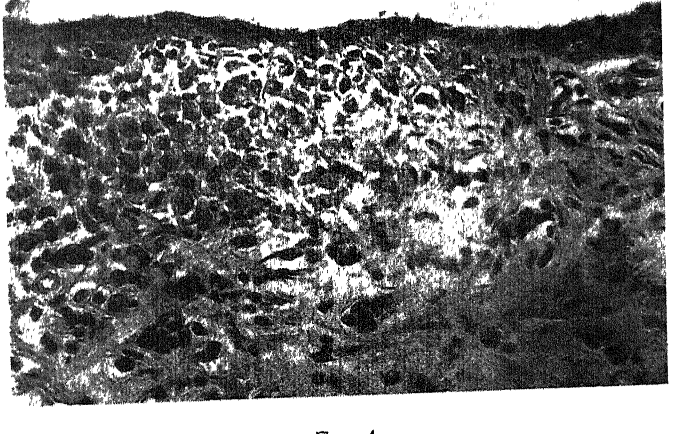

FIG. 4

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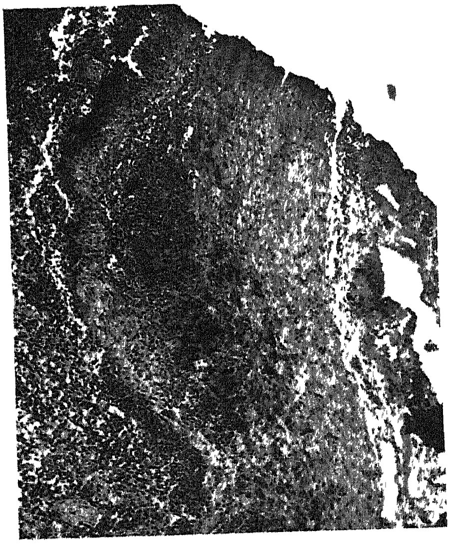

FIG. 5

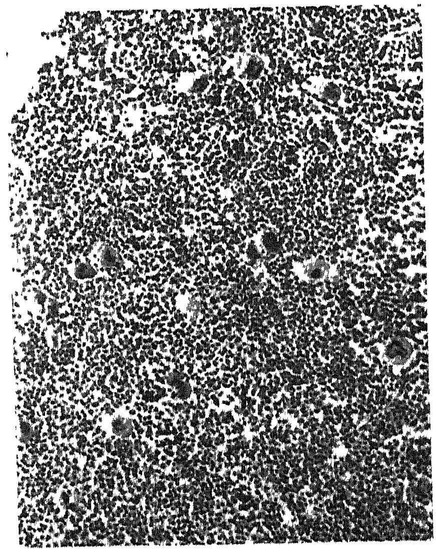

FIG. 6

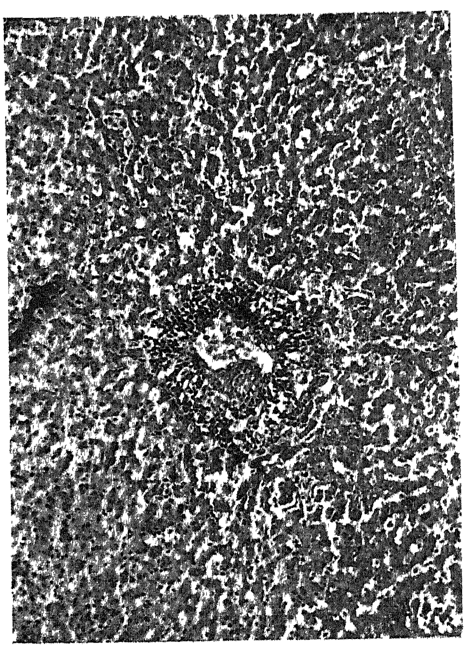

FIG. 7

FIG. 8

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Investigations on Mediterranean Kala Azar

VIII—Further Observations on Mediterranean Sandflies

By S. ADLER and O. THEODOR (Kala Azar Commission of the Royal Society)

(*Communicated by Sir Henry Dale, Sec. R.S.—Received June* 4, 1934)

MALTA

The sandflies of Malta have been previously described by Marett (1910), Newstead (1911), and Whittingham and Rook (1922). The attention of these authors was attracted mainly to *Phlebotomus papatasii* which they considered to be the commonest sandfly on the island.

The observations recorded in the present paper cover the whole of the sandfly seasons of 1931 and 1932. The following six species were found:—

P. perniciosus Newstead.
P. papatasii Soop.
P. parroti Adler and Theodor (formerly considered as *P. minutus*).
P. major Annandale.
P. sergenti Parrot.
P. macedonicus Adler and Theodor.

The last three species are new for Malta. *P. macedonicus* was found in small numbers in a limited area on the island of Gozo and this species is too rare locally to play any part in the transmission of disease. *P. sergenti* is also relatively rare and irregularly distributed and is apparently of no great local importance. The same applies to *P. major*, which is more common than *P. sergenti*, but is not sufficiently numerous to play an important part in the transmission of the disease. *P. papatasii* is widely distributed and has attracted considerable attention owing to its relationship to sandfly fever.

Our main attention was fixed on *P. perniciosus* which is by far the commonest sandfly in Malta and Gozo. It is at least twenty times as numerous as *P. papatasii*, but it has not attracted as much attention as the latter sandfly because it is not found in large numbers in dwellings during the daytime.

Newstead (1911) suggested the possibility of *P. perniciosus* being a vector of sandfly fever. Actually the distribution of sandflies in Malta provides very good evidence against this possibility. In spite of the fact that many cases of sandfly fever among the troops are sent to the military hospital at Intarfa, the disease is almost unknown among the personnel of the hospital and their families. Major Walker, R.A.M.C., kindly collected sandflies in the hospital and the adjacent houses. Of 60 specimens collected all were *P. perniciosus*; *P. papatasii* is either rare or absent.

Distribution and Bionomics

P. perniciosus makes its appearance in Malta about the beginning of May. They are rare after the beginning of November, but specimens were caught as late as November 18, 1932. (In 1931 no sandflies were observed after November 5). Professor W. Bernard has found isolated specimens as late as the middle of December, and our observations on the hatching of pupæ kept at room temperature during November and December confirm his findings. After the beginning of November the sandfly is too rare to play a significant role in the transmission of disease.

Although *P. perniciosus* is ubiquitous in Malta there are certain streets in which the conditions for breeding are particularly favourable and where the sandfly occurs in such vast numbers during the sandfly season that it is possible to catch two to three hundred unfed females in an hour on a piece of wall about 10 metres long. These localities of maximum infestation are apparently associated with a good supply of moisture which favours oviposition and they occur near public water taps, moist cellars, drains and gutters from which sandflies were seen to emerge.

It was not possible as in Sicily to correlate the distribution of this species to that of infantile Leishmaniasis, for both the sandfly and the disease are of almost uniform distribution, but cases are much commoner in areas of dense infestation with *P. perniciosus* than in other parts.

The bionomics of this species have already been discussed in a previous paper. It is only necessary to add that *P. perniciosus* is an out-of-door species to a greater extent than was realized during our work in 1930. Although it enters houses freely, far more specimens can be found outside houses than in living rooms. Many sandflies do not enter houses at all, but feed on dogs and human beings in the streets and then disappear in cracks near the bottom of walls.

As in Catania, this species was found to be distinctly zoophilic and to feed far more readily on cows and dogs than on man. Not more than 10% of unfed wild sandflies feed on man in the laboratory while

50% or more feed on dogs. Laboratory bred sandflies feed so seldom on man or hamsters that it is hardly practical to attempt feeding experiments with them on a large scale, and, since it is so easy to capture large numbers of unfed wild females, most of our experiments were carried out with wild sandflies.

As previously stated, *P. perniciosus* seldom re-feeds until it has laid a batch of eggs. In the laboratory it is possible to distinguish between females with an empty alimentary tract which have laid eggs prior to re-feeding, and those which have never fed by an examination of the sebaceous (accessory ovarial) glands. In newly hatched females the sebaceous glands are empty and the typical granules never appear unless the sandfly has had a blood meal; even a small feed is sufficient to cause the appearance of the typical granules. A few days after feeding the sebaceous glands are full of granules. During egg laying the majority of the granules disappear and they are probably passed out with the eggs, but a varying number remain and can easily be seen during dissection. The presence of these granules in the sebaceous glands of females with an empty alimentary tract is the only morphological feature which distinguishes females which have survived egg laying and are due for a re-feed from newly hatched ones, and judged by this criterion less than 5% of wild unfed females have laid eggs. Occasionally *P. perniciosus* re-feeds even when a few apparently ripe eggs are left in the abdomen.

The development of eggs depends on two factors, fertilization and a blood meal. In unfed females the eggs contain no yolk granules, and there is no appreciable growth of the eggs.

In fully gorged unfertilized females yolk granules appear in the eggs which grow till they are as broad, but only about half as long as normal ripe eggs. The rate of development of eggs in a fertilized and fully fed female depends on the temperature ; at 27° C the eggs are ripe in four or five days (at 30° C in three to four days).

The appearance of the granules in the sebaceous glands is independent of copulation and depends only on a blood meal.

Method of feeding Sandflies in the Laboratory

Wild unfed sandflies are placed in a small sandfly proof gauze cage attached to the thigh of a dog. Within 15 to 20 minutes many of the insects feed and nothing is to be gained by longer exposures.

It is not possible to adopt a similar method with hamsters or spermophils on which *P. perniciosus* does not feed as readily as on dogs. A small sandfly proof gauze cage about 15 cm in every dimension is placed within

a larger sandfly proof cage about 30 cm in every dimension. A small wire cage containing a hamster or spermophil is placed within the inner gauze cage into which the unfed sandflies are introduced and both cages are then closed. Prior to the feeding experiment the hair is removed from a part of the animal's back. The sandflies are introduced into the feeding cage as soon as possible after their capture in the late hours of the evening. On the following morning the hamster is removed from the feeding cage and the sandflies are caught and separated into three groups: fully fed, slightly fed, and unfed. The first group are used for transmission experiments, the two other groups are given an opportunity of re-feeding, the slightly fed group after a few days, and the unfed group on the following evening.

It is to be noted that after a number of exposures both Chinese hamsters and spermophils learn to destroy sandflies and therefore only a varying number of sandflies complete their feed; thus thirty fully fed females out of a batch of one hundred introduced into a feeding cage is considered a very satisfactory result and usually not more than 15% succeed in taking a full fed on an " educated " animal.

Breeding technique

During 1930 many of the caught, fully gorged *P. perniciosus* did not lay eggs, some laid only a part of their eggs, very few laid a full batch of eggs and none survived egg laying. During 1931 and 1932 a special study on the optimum conditions for egg laying was made and it was found that relative humidity is the deciding factor in oviposition. Small gauze cages containing gorged sandflies were placed in air-tight glass vessels in which the humidity was regulated by varying proportions of sulphuric acid and water. It was found that whereas egg development was not influenced to any marked degree by relative humidity (eggs developed normally when the insects were kept at a relative humidity of 70%), the insects laid all or nearly all their eggs only when the relative humidity approached 100%. Parallel experiments with similar results were carried out in breeding pots and vessels in which the humidity was measured by a small calibrated hair hygrometer which Professor P. A. Buxton, of the London School of Tropical Medicine, presented to us. This instrument proved to be very useful in spite of the fact that it required calibration at frequent intervals, and does not give accurate data at very high humidities.

P. perniciosus is far more dependent on the surrounding relative humidity than *P. papatasii*, probably because the latter sandfly can

compensate for the loss of moisture by frequent re-feeds prior to oviposition, but even *P. papatasii* will lay all its eggs after a single feed and occasionally survive and re-feed if kept at a relative humidity of 100% after gorging.

After numerous trials the following technique was finally adopted. It is based on the assumption that since *P. perniciosus* feeds once before egg laying and then develops a full batch of eggs (14 to 70) without further access to fluid, it must be guarded against loss of moisture which can only occur at the expense of tissue fluid and not at the expense of the developing eggs (the latter apparently develop normally independently of the relative humidity).

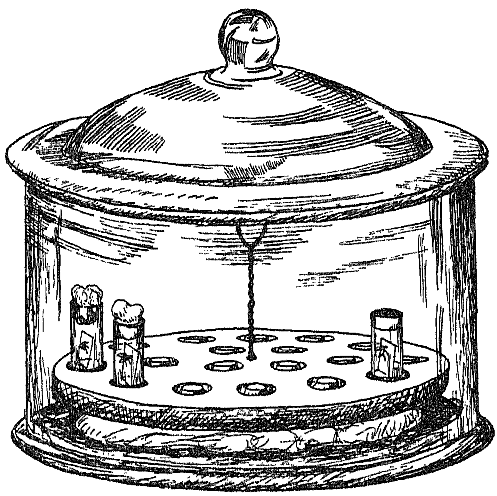

Fig. 1—Method of keeping sandflies for egg laying

A circular slab of porous Maltese stone about 1 cm thick and 20 cm in diameter is prepared and a number of shallow circular grooves 1·5 cm in diameter are carved in the surface. The stone is placed in water and allowed to absorb moisture to its maximum capacity and is then left to dry in air till the film of surface moisture has disappeared. Fully gorged sandflies caught in the feeding cage are placed in glass cylinders 1·5 cm in diameter and about 4 cm in height. A strip of blotting paper is introduced into the glass cylinder which is closed at one end with absorbent cotton-wool or gauze and the other end is fitted into a groove on the stone and kept securely in position by a circular strip of plasticine previously moulded into the groove. When all the grooves are occupied by glass cylinders containing sandflies the stone is placed in a glass vessel containing a little moist wool, the vessel is closed with a tightly fitting lid and kept in a dark room, fig. 1.

Under these circumstances each sandfly is assured of a relative humidity approaching 100%.* Considerable experience is necessary in order to gauge the extent to which the stone is allowed to dry before being placed in the glass vessel. Each glass cylinder must be thoroughly cleaned, otherwise drops of moisture to which the sandflies adhere appear on the surface. A constant temperature is not essential within certain limits, although it is desirable as it tends to eliminate the deposition of dew on the glass cylinders. The glass vessel is opened daily and its contents examined. After a number of days (depending on temperature) the fertilized sandflies lay eggs mainly on the blotting paper and partly on the stone. After oviposition each sandfly is removed and given an opportunity of re-feeding. Under the above conditions about 80% of sandflies lay all their eggs and from 10% to 80% survive egg laying. After counting the number of eggs the strips of blotting paper are placed in a breeding pot.

Hibernation in P. perniciosus

It was found in 1930 that hibernating larvæ appeared in broods of *P. perniciosus* from Catania towards the end of the sandfly season. Many of these larvæ (up to 90%) continued hibernating in spite of being kept at 30° C for 14 weeks. This phenomenon was observed as early as the end of August, but was most marked in later broods starting at the end of September and in October. It was pointed out that whatever its origin, hibernation in *P. perniciosus* is apparently a cyclical phenomenon which cannot be explained by changes in temperature only.

These observations were repeated in 1931 and 1932. Hibernating larvæ were observed in August, at an average temperature of 28° C, in broods in which the majority of the larvæ developed actively.

Hibernation was still more marked from the middle of September onwards. In 1931 the average temperature during the development of the three first larval stages at this time of the year was between 22° and 24° C. Fourth stage larvæ appeared at a temperature of 22° C and the average temperature during the first ten days of the fourth stage larvæ was 21° C. In these broods all larvæ hibernated. A rise of temperature up to 25·5° C during six days, after the larvæ had been kept at 20° to 22° C for ten days, did not induce the larvæ to active development. During September and the first half of October, 1932, the temperature was about 27° C during the whole time of development of the larvæ.

* At a relative humidity approximating to 100% unfed laboratory females live up to five days and males up to eleven days.

A number of larvæ developed actively, and a varying number (from 20% to 70%) hibernated. It appears therefore that towards the end of the sandfly season there is practically no active development of the larvæ at a temperature of 20° to 22° C.

Observations in Malta and Catania show that sandflies disappear from the beginning to the middle of November when the average temperature is between 16° and 21° C.

In the middle of November broods were shipped to Jerusalem where they were kept at a room temperature of 14° to 16° C in winter. A few sandflies hatched during December and January, *i.e.*, they did not hibernate, but their rate of development was retarded corresponding to the low temperature. The majority remained in hibernation up to the beginning of April when pupation commenced. Hatching commenced on May 2, 1932, and continued up to the end of June. Larvæ which were kept during winter on top of an ice chest hatched two to three weeks later than those kept at room temperature. It is to be noted that after hibernation the pupal stage in some broods lasted about thirty days at room temperature (in Jerusalem about 20° C in April). Sandflies appeared on May 17, 1932, in Malta, and it therefore seems that the development of hibernating larvæ at room temperature approximates to that occurring under natural conditions.

Larvæ from broods commenced in the middle of September which entered the winter as fourth-stage larvæ pupated and hatched at the same time as those from broods in which the eggs were laid at the end of October and the beginning of November. The latter developed slowly up to the fourth stage and then remained resting until they emerged at the beginning of May simultaneously with the September larvæ. The duration of hibernation apparently does not within certain limits influence the beginning of active development.

It follows from the above observations that the first generations of wild sandflies which appear at the beginning of the sandfly season are a heterogeneous group. They include sandflies which originate from eggs laid in August of the previous year as well as those from eggs laid towards the middle of November.

Effects of high temperatures on hibernating larvæ.—Broods of larvæ which had been standing at room temperature were placed in an incubator at 30° C at various dates. In 1931 some broods were placed at 30° C on December 4. A number of larvæ pupated and hatched, but the mortality among the pupæ was very high in some broods. Some larvæ did not pupate even after being kept eight weeks at 30° C. In 1932 pots were placed at 30° C in the beginning of February and the large

majority of larvæ pupated and hatched. The length of the pupal stages, however, was prolonged up to 18 days in some cases as against 7 to 9 days in normal active broods at 30° C.

During 1931 and 1932 mixed layings from 25 to 30 females were observed. Exact results can only be obtained by observing the offspring of single females, but this was impossible under the conditions in which the work was carried out.

The following facts appear from the above observations:—

1. Hibernation starts in August in spite of a mean temperature of 27° to 28° C at which there is normal development in the earlier generations.
2. Hibernation is most marked between September and November even if the average temperature during the development of the fourth-stage larva is about 27° C.
3. Below a temperature of 21° C there is practically no active development. Rising the temperature up to 25·5° C for 6 days after keeping the larvæ 10 days at 21° C does not induce active development.
4. A relatively small number of larvæ do not hibernate even at low temperature (15° to 20° C). This explains the occasional finding of sandflies in December.

P. parroti

This sandfly was far more numerous in 1931 than in 1932. It was collected mainly for feeding experiments on the gecko *Tarentola mauretanica*, for a study of the life-cycle of *Trypanosoma platydactyli*. This sandfly was observed to feed only on geckoes on which it takes as long as two hours or even more to complete its feed. The animal makes no attempt to interfere with the unfed sandfly, but in the laboratory several fully gorged females were apparently swallowed by geckoes. (Feeding experiments were carried out in glass flasks closed with a tightly fitting wool stopper.)

P. parroti usually re-feeds once before laying eggs, but it can lay a full batch of eggs after a single feed.

This sandfly was bred in the laboratory by the usual technique used for *P. papatasii*.

CATANIA

Additional observations on *P. perniciosus* in Catania were made in August and September, 1931, and from July to November, 1932. The

prevalence of *P. perniciosus* in Catania was considerably underestimated during 1930, for it was not recognized at that time that at suitable hours this sandfly can be more readily observed and captured out of doors than in houses. Thus in 1930 the average number of unfed females caught per evening in a house in the sandfly belt of Catania were 4 in July, 20 in the second half of August and 70 in the second half of September. During 1932, on the exterior of the same house and the immediate neighbourhood, the numbers caught were 100 for 2 hours in July, 120 in August, and 200 in September. Sandflies became rare towards the end of October, 1932 (while they were still numerous in Malta), and the sandfly season terminated on November 5, *i.e.*, several weeks before Malta.

Greece

Observations were carried out for a short period of three weeks in August, 1932, and sandflies were collected in Athens, Kavallah (Macedonia), and Argos. It is therefore impossible to give an account of the bionomics and seasonal distribution of Greek sandflies, but several of the findings are suggestive.

In Kavallah the following species were found: *P. papatasii*, *P. sergenti*, *P. macedonicus*, *P. perniciosus* var. *tobbi*, and *P. major*. The first four species are common while *P. major* (4 ♀ ♀) were found only once in a dried and deserted well about three hundred yards from the nearest human habitation. It is to be noted that relatively few cases of visceral or cutaneous Leishmaniasis have been recorded from Macedonia.

In Athens the following species were found: *P. papatasii*, *P. sergenti*, *P. major*, *P. parroti* and *P. minutus*. *P. major* was particularly common in the neighbourhood of the Pasteur Institute where, according to the director, Dr. Lépine, several cases of infantile Leishmaniasis have recently been noted. The latter sandfly is the most probable vector of visceral Leishmaniasis in the city. No specimens of *P. perniciosus* were found, although Cardamatis (1931) records a single specimen which he raised from a larva found in stagnant water (?). It has already been shown (1931) that *P. major* becomes infected more readily than *P. perniciosus* with *L. infantum* by feeding on dogs.

The findings in the vicinity of Argos were instructive. Infantile Leishmaniasis is hardly known in Argos, but it is common in the neighbouring hills. Very few sandflies (*P. papatasii* 1 ♀, and *P. sergenti* 1 ♀) were found in Argos. Owing to the kindness of Dr. C. Marinos, the Medical Officer of Argos, an examination was made of the neighbouring

village of Sikkoria (250 metres above sea level, population 400). This village was selected because three cases had been diagnosed within the previous twelve months. Two species of sandflies were found : *P. major* (46 ♂ ♂ and 15 ♀ ♀ in one hour) and *P. chinensis* var. (1 ♀). It is to be noted that in the neighbourhood of Argos infantile Leishmaniasis is mainly a disease of the hills and most of the cases occur between 150 to 600 metres above sea level. The incidence of the disease in the hill districts round Argos is far higher than that of Catania or Malta and according to Dr. Marinos was recently found to be about 30 per 4000 inhabitants annually.

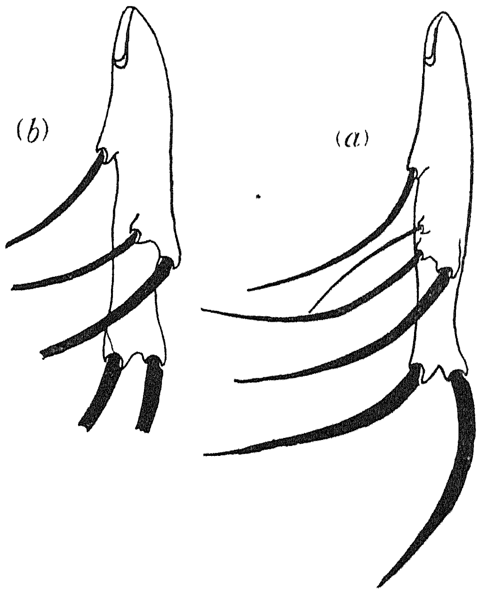

Fig. 2—(*a*) Supernumerary spine on distal segment of right superior clasper of *P. major*. (*b*) Distal segment of left clasper of the same specimen

One specimen of *P. major* captured in Athens was found to have an interesting abnormality in the form of a supernumerary spine on the distal segment of the right superior clasper (fig. 2). The supernumerary spine was about half the length and thickness of the small normal median spine and was situated slightly proximal to the two median spines. The arrangement of spines on the left side was normal. Supernumerary spines on the superior clasper have been described by Sinton (1927) in *P. major* and other species and Parrot (1921) in *P. perniciosus*.

Newstead (1914) differentiated *P. mascittii* Grassi from *P. perniciosus* only on the basis of a sixth spine on the distal segment of the superior clasper. It is very probable that Newstead was dealing with an abnor-

mality such as the present one for in other respects his drawings of *P. mascittii* correspond to those of *P. perniciosus*.

PALESTINE

An account of Palestinian sandflies has already been given elsewhere (1930). During the summer and autumn of 1933 another species, viz., *P. macedonicus*, was found to be very common in the valley of Jezreel. From 1926 to 1929 collecting in Palestine failed to reveal this species probably because of its peculiar bionomics. Whereas the other Palestinian species of the major group appear a few hours after sunset *P. macedonicus* appears in large numbers during the summer and autumn towards midnight. The female is very similar to that of *P. perniciosus*, from which it can be distinguished in mounted specimens by slight differences in the pharyngeal armature and in fresh-dissected specimens by the sebaceous granules which are smaller and more coccoid than those of *P. perniciosus*.

This species was the only member of the major group found in the vicinity of a house where a case of infantile Leishmaniasis was contracted and is therefore to be regarded as a probable carrier, although not a very effective one, for the disease is very rare in Palestine. Since 1928 only five cases of locally acquired infantile Leishmaniasis have been found in Palestine of which two occurred in the valley of Jezreel where *P. macedonicus* is the only known species of the major group and one in Jerusalem in a district where *P. major* var. *syriacus* is common.

It is to be noted that *P. macedonicus* has a wide distribution and is known from Greece, Malta, Palestine, and Hungary.

Feeding experiments with Palestinian sandflies will be described in another communication.

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Investigations on Mediterranean Kala Azar

IX—Feeding Experiments with *Phlebotomus perniciosus* and other species on Animals infected with *Leishmania infantum*

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(*Communicated by Sir Henry Dale, Sec. R.S.—Received June* 18, 1934)

In the present paper it is intended to analyse the results of feeding experiments with *Phlebotomus perniciosus* on animals infected with various strains of *Leishmania infantum* carried out during 1930, 1931, 1932 and 1933.

In a previous paper (1931) the results of feeding experiments carried out on a Chinese hamster from June 18 to October 22, 1930, were discussed. Only 330 sandflies infected by feeding on a Chinese hamster inoculated with Catania strains of *L. infantum* were included, and the results obtained appeared fairly uniform but for one point. A large majority of sandflies had an infection in which flagellates described as "long forms" predominated and only a small proportion (5%) were infected with "short forms." Proboscis infections (*i.e.*, infections in the epipharynx) were observed mainly among the latter. The "short form infections" were observed from September 20 till the end of the sandfly season in 1930.

No particular attention was paid to the above peculiarities because it was not till the end of August, 1930, that a good method of keeping *P. perniciosus* in captivity was devised.

In general the results of the feeding experiments appeared uniform in so far as practically all the infected sandflies showed an infection of the upper part of the cardia two days and more after the infecting feed. Infected sandflies without flagellates in the upper part of the cardia were either not observed or were so few that they did not attract attention. The relative rapidity with which flagellates ascended up the œsophageal valve was most striking and the pharynx was invaded as early as three days after the infecting feed.

FEEDING EXPERIMENTS IN 1931

During 1931 feeding experiments were commenced in Malta on animals infected with Maltese and Catania strains and it was naturally expected that the results obtained during the previous year in Catania would be repeated. It was therefore surprising to find, particularly in sandflies infected with Maltese strains, a relatively wide variation in the distribution of flagellates in the alimentary tract of *P. perniciosus*. Feeding experiments were repeated in 1932 and on a smaller scale in 1933 with a similar variation in the distribution of flagellates.

Animals used for Feeding Experiments

(1) Chinese hamsters for which we are indebted to Dr. Earle of the Lester Institute for Medical Research, Shanghai, and to the Royal Society.

(2) Syrian hamsters, *Cricetus auratus*, bred in the laboratory in Jerusalem.*

(3) Spermophils, *Citillus citillus*, for which we are indebted to Dr. Caminopetros of the Pasteur Institute, Athens.

(4) Naturally infected dogs caught in Malta.

In Chinese hamsters a visceral infection becomes established as early as three weeks after inoculation, and skin infection sufficiently intense for infecting sandflies about six weeks after inoculation, but this varies in individual animals.

Syrian hamsters are not as satisfactory as the Chinese animals, for a suitable skin infection does not appear till about eight months after inoculation, *i.e.*, about two months prior to the death of the animal as a result of a very intense fatty degeneration of the liver which is characteristic of infections with *L. infantum* in *Cricetus auratus*. In advanced infections depilation and seborrhea occur as in naturally infected dogs. The longevity of infected Syrian hamsters depends on the strain of flagellates used, *e.g.*, animals infected early in 1933 with an Indian strain of *L. donovani* maintained in cultures in the laboratory since 1927, appeared in excellent condition in February, 1934, in spite of a visceral infection, but with recently isolated strains of Mediterranean *L. infantum* the majority of the animals succumb in nine to ten months.

* We have to thank Mr. H. Ben-Menahem of the Hebrew University, Jerusalem, who determined the conditions for breeding *C. auratus* under laboratory conditions and was able to produce a continuous supply of these animals.

The spermophil is the most susceptible of all animals used so far; a skin infection is established as early as 15 days after inoculation but an infected animal only lives for a few months.

The total number of infected *P. perniciosus* was 3635 and the total number of infected animals used was 40. The above figures are sufficiently large to allow an opinion on the various factors involved, individual variation in different animals, variation in sandflies, seasonal differences, etc. In addition some of the experiments were controlled by using sandflies from different sources, *e.g.*, both Catania and Maltese sandflies were fed on the same animal and the results compared.

The distribution of flagellates in infected sandflies on the fourth day or later after the infecting feed may be as follows:—

(1) The infection is confined to the stomach.

(2) The infection involves the stomach and lower parts of the cardia.

(3) The infection reaches up to the foremost parts of the cardia.

(4) The infection proceeds anteriorly as far as the teeth of the epipharynx.

In all these types of infection the hindgut may or may not be involved. (Hindgut infections are comparatively rare.)

The condition of the pharynx and buccal cavity is not included, for this can only be determined by special dissection or by examination of sectioned specimens.

In the above classification only sandflies examined four days and more after the infecting feed are included. It was found that after four days the distribution of flagellates in the infected sandflies is not appreciably affected by time, *e.g.*, proboscis infections were found as early as four days after the infecting feed, and the distribution of flagellates in sandflies dissected eight days after the infecting feed was nearly the same as in those dissected after four days. The intensity of infection did not appreciably influence the type of distribution; intense infections were found in some cases when the flagellates were restricted to the stomach and every degree of intensity was found in every type of distribution, although there was a larger proportion of slight infections in cases where the flagellates were confined to the stomach.

Malta Strains—The following are examples of the results of feeding experiments with *P. perniciosus* on animals infected with Maltese strains.

Spermophil No. 2—Between June 22 and July 15, 1931, inoculated intraperitoneally with 9·5 cc of culture of *L. infantum*. Feeding experi-

ments commenced on July 14 and gave positive results on July 15, 1931.

Between July 15 and August 21, 1931, 88 *P. perniciosus* fed on this animal; of these 72 took a full feed and 16 a partial feed. Forty-four sandflies became infected. Between July 15 and July 23, 1931, the infection rate in fully gorged sandflies was about 25% and subsequently rose to 90%. During the course of the experiment the types of the infection in the sandflies did not change.

TABLE I—SHOWING TYPES OF INFECTION IN *P. perniciosus* FED ON SPERMOPHIL NO. 2

No. of days after feed	No. of infected sandflies	Remarks
4	2	Flagellates in stomach and the lower part of cardia.
5	10	In 3 the infection was confined to the stomach. In the remainder cardia also infected.
6	13	In 3 infection confined to the stomach. In the remainder cardia also infected.
7	7	In 3 infection confined to the stomach. In 1 lower part of cardia infected, in the remainder flagellates extending up to anterior part of cardia.
8	8	In 1 infection confined to the stomach. In 3 lower part of cardia infected. In the remainder anterior part of cardia infected.
9	1	Stomach and cardia infected.
10	3	In 1 infection confined to the stomach. In 2 stomach and cardia infected.

In about 25% of the infected sandflies the infection did not extend beyond the anterior part of the stomach.

Spermophil No. 1—Inoculation as in No. 2. Feeding experiments with *P. perniciosus* were commenced on July 8 and first gave positive results on July 28, 1931. Between July 28 and August 26, 1931, 94 sandflies fed on this animal and 40 became infected. Up to August 14 the infection rate was about 30% and subsequently rose to 75%.

As with spermophil No. 2, in about 25% of the sandflies the infection did not extend beyond the anterior part of the stomach. In the case of infections in the upper part of the cardia there were instances in which the flagellates were not attached to the rhabdorium but were free in the lumen. It may be noted that temperature (between 21° C and 35° C) did not affect the type or course of the infection in the sandfly. This

applies to the whole of the feeding experiments carried out between 1930 and 1933.

The variability of the types of infection in *P. perniciosus* was most striking and the frequency of infections confined to the stomach or to the stomach and lower part of the cardia was such that it was thought that the main carrier in Malta might be a sandfly of the *major* group other than *P. perniciosus*, but *P. major* is too rare to play an important part and an exhaustive search revealed only a few specimens of *P. macedonicus* in Gozo.

TABLE II—SHOWING TYPES OF INFECTION IN *P. perniciosus* FED ON SPERMOPHIL NO. 1.

No. of days after feed	No. of infected sandflies	Remarks
4	1	Infection confined to stomach.
5	1	,, ,,
6	12	In 5 infection confined to stomach, in 3 lower part of cardia also infected, in 4 anterior part of cardia infected.
7	10	In 1 infection confined to stomach, in 1 lower part of cardia also infected, and in 8 anterior part of cardia also infected.
8	2	In 1 infection extended to anterior part of cardia, and in 1 to lower part of cardia only.
9	12	In 2 infection confined to stomach, in 1 lower part of cardia also infected, and in 9 anterior part of cardia also infected.
10	1	Cardia and stomach infected.
12	1	,, ,,

Chinese Hamsters

Inoculation of Chinese hamsters with Maltese strains of *L. infantum* was commenced on June 29 and it was not till August 19, 1931, that feeding experiments on one animal (No. 10) gave positive results, *i.e.*, after the feeding experiments on the spermophils were completed. (The latter were not continued systematically because only a very small proportion of sandflies fed on these animals.)

The bulk of the experiments was carried out on Chinese hamster No. 10 because with this animal a high infection rate was obtained from the beginning.

Chinese Hamster No. 10—Between June 29 and August 8, 1931, received 7·5 cc of *L. infantum* from Malta intraperitoneally. August 8, 1931, a piece of spleen was removed and found heavily infected. Feeding

experiments with *P. perniciosus* first gave positive results on August 19, 1931. Out of 609 *P. perniciosus* from Malta fed on this animal and dissected between August 19 and November 2, 1931, 526 were found positive. (Over 2000 were given an opportunity of feeding.) The results of the dissections are of sufficient interest to be tabulated in detail particularly as the types of infection change very strikingly towards the end of the experiment.

TABLE III—RESULTS OF FEEDING EXPERIMENTS WITH MALTESE *P. perniciosus* ON CHINESE HAMSTER NO. 10

Date fed	Total fed	Total positive	Remarks
August 19 ..	2	2	In 1 cardia only infected, in 1 stomach and cardia.
,, 20 ..	3	2	Stomach and cardia infected.
,, 22 ..	1	1	Stomach and cardia near œsophageal valve.
,, 26 ..	16	12	In 2 stomach only infected, in 4 lower part of cardia, in 6 infection extending up to anterior part of cardia.
,, 28 ..	15	13	In 1 stomach only, in the other stomach and cardia infected.
,, 29 ..	46	27	In 3 stomach only, in 3 lower part of cardia, remainder up to anterior part of cardia.
September 1	6	5	In 1 stomach only, in 1 lower part of cardia involved, in remainder anterior part of cardia infected.
,, 4	6	3	Stomach and cardia infected.
,, 17	8	7	In 3 stomach only, in remainder up to anterior part of cardia.
,, 20	4	4	In 1 stomach only, in 3 up to anterior part of cardia.
,, 23	4	2	In 1 stomach up to middle of cardia, in 1 anterior part of cardia.
,, 29	17	11	In 1 after 3 days stomach only, in 2 stomach and lower part of cardia, in 8 anterior part of cardia.
,, 30	18	17	In 1 heavy infection in stomach and a few flagellates in cardia, others with massive infections in cardia.
October 2 ..	15	14	In 1 after 2 days in stomach only, in 1 up to middle of cardia, in others anterior part of cardia.
,, 6 ..	4	4	Stomach and anterior part of cardia.
,, 7 ..	35	34	In 3 stomach only, in 2 few flagellates also in cardia, others anterior part of cardia.

TABLE III—(continued)

Date fed	Total fed	Total positive	Remarks
October 8 ..	18	17	In 2 stomach only, in 2 up to middle of cardia.
,, 9 ..	23	23	In all up to anterior part of cardia.
,, 10 ..	27	26	In 1 after 3 days stomach only, in others anterior part of cardia infected, and 1 *with long forms in teeth of epipharynx.*
,, 11 ..	16	15	In all top of cardia.
,, 12 ..	15	15	,,
,, 13 ..	12	11	,,
,, 14 ..	18	15	In 1 up to middle of cardia. In others anterior part of cardia. In 1 *tip of proboscis.*
,, 15 ..	15	15	In all top of cardia. In 1 cardia choked and stomach negative. *In 1 short forms.*
,, 16 ..	6	6	In all top of cardia. In 1 stomach negative and in 1 only a few flagellates in stomach.
,, 17 ..	15	15	In 1 a single flagellate was found in the stomach, in others in the top of cardia.
,, 18 ..	27	24	In all anterior part of cardia.
,, 19 ..	19	17	In all top of cardia. In 1 stomach negative.
,, 20 ..	6	6	In 1 stomach only, in others top of cardia. *In 1 proboscis infected.*
,, 21 ..	19	18	In all top of cardia, *in 1 short forms.*
,, 22 ..	19	16	In all top of cardia.
,, 23 ..	20	18	2 after 2 days in middle of cardia, others in the top of cardia. *In 5 short forms, in 1 short forms in teeth of epipharynx.*
,, 24 ..	14	11	In all top of cardia. *In 2 short forms. In 1 hundreds of short forms in teeth of epipharynx.*
,, 26 ..	27	27	In 1 stomach only. Others top of cardia. *In 3 short forms in œsophagus of which 1 had a proboscis infection.*
,, 28 ..	30	26	In all top of cardia. *In 1 short forms.*
,, 29 ..	5	5	In all top of cardia.
,, 30 ..	8	8	In all top of cardia. In 1 *proboscis infection with short forms.*
November 1..	13	13	In all top of cardia. In 2 *proboscis infections.*
,, 2..	5	4	In all top of cardia. *In 1 short forms.*

An analysis of Table III brings out the following facts :—

1. There is a large variation in the distribution of flagellates in individual sandflies fed at the same time on the same animal. Thus out of a batch of five sandflies which fed on Chinese hamster No. 10 on September 1, the flagellates were confined to the stomach in one, reached up to the

lower part of the cardia in one and to the upper part of the cardia in the remainder.

2. Differences in the distribution of flagellates do not depend on the intensity of infection.

3. From the second week of September onwards the results of the feeding experiments became more uniform in that the overwhelming majority of sandflies showed an infection which reached at least as far as the upper part of the cardia. The tendency to an anterior position in the sandfly became more marked and there were instances where flagellates were few or absent from the stomach, while the anterior part of the cardia was infected. The flagellates ascended more rapidly than in the earlier experiments and the anterior part of the cardia was reached as quickly as two days after the infecting feed.

"Short form" infections with flagellates 4 to 10 μ long and with a flagellum usually longer than the body are a very striking phenomenon and are in marked contrast to the usual "long form" infections in which the flagellates are up to 35 μ long.

The "short form" infections are rare and were only found 13 times out of a total of 239 sandflies which infected themselves between October 15 and November 2, 1931. Infections in the epipharynx are relatively much commoner in the "short form" infections than in the other types (4 out of 13 as against 3 out of 308 "long form" infections).

Feeding experiments were carried out on other Chinese hamsters infected with Maltese strains but the results are not of the same interest as those recorded above for although the animals were inoculated at the same time the skin infection appeared later.

TABLE IV SHOWING FEEDING EXPERIMENTS WITH *P. perniciosus* ON CHINESE HAMSTERS NOS. 8, 9, 11 AND 16 IN 1931

Date	Total fed	Total positive	Remarks
			Chinese hamster No. 8
September 3	8	2	In 1 stomach and lower part of cardia, in 1 anterior part of cardia.
,, 14	9	2	In 1 stomach only, in 1 anterior part of cardia.
			Chinese hamster No. 9
September 3	23	13	In 1 stomach only, in 1 middle of cardia, in 11 anterior part of cardia.
,, 11	4	2	In stomach only.
,, 13	11	8	In 1 stomach only, in 7 anterior part of cardia.
,, 17	5	5	In 1 stomach only, in 4 anterior part of cardia.

TABLE IV—(continued)

Date	Total fed	Total positive	Remarks
			Chinese hamster No. 11
August 26 ..	10	1	Many flagellates in stomach, few in cardia.
September 3	17	4	In 1 stomach only, in 2 middle of cardia.
,, 13	6	2	Stomach only.
October 14 ..	12	12	In 1 middle of cardia, in others anterior part of cardia in 1 infection almost confined to cardia.
,, 15 ..	6	3	In all anterior part of cardia.
,, 22 ..	2	2	Middle of cardia.
,, 30 ..	8	8	In all anterior part of cardia, in 1 *infection of short forms in epipharynx.*
			Chinese hamster No. 16
September 15	2	1	Anterior part of cardia.
,, 16	6	1	Stomach only.

As will be seen from Table IV, the same variability in the types of infection noted in Chinese hamster No. 10 recurred. In the case of hamster No. 11 on which feeding experiments were continued till the end of the sandfly season the same peculiarity noted in No. 10 was observed, *i.e.*, after the second week in September the infections became more frequently anterior in character.

Catania Strains

Chinese Hamster No. 2—Inoculated 1930 with cultures of *L. infantum* and infected human bone marrow. Feeding experiments with *P. perniciosus* carried out on a small scale in 1930 gave negative results. Feeding experiments were carried out continuously from May 24 to September 6, 1931, and during this period 331 sandflies out of a total of 388 dissected were found infected. It is not necessary to tabulate the results for they were strikingly uniform throughout the whole course of the experiments with respect to the infection of the anterior part of the cardia. Only four sandflies showed slight infections of the cardia accompanied by massive infections of the stomach and there was not a single instance of an infection confined to the stomach four days or more after feeding. Five sandflies showed proboscis (epipharynx) infections and these fed on June 11 and 18, August 23, September 4 and 6, 1931. Only three sandflies had " short form " infections and of these two were infected in the epipharynx.

Chinese Hamster No. 26—Inoculated August 6, 1931, intraperitoneally with flagellates from sandfly No. 478 infected by feeding August 2 on Chinese hamster No. 2.

September 27, 1931—A piece of spleen was removed and found infected. Feeding experiments were carried out on October 28 and 29, 1931. Out of 41 sandflies found infected three days or more after feeding one had a stomach infection (a single flagellate) and in all the others the anterior part of the cardia was infected. Two sandflies had proboscis infections (long forms).

In order to determine whether there is any appreciable difference between the behaviour of *L. infantum* in Malta and Catania sandflies two Chinese hamsters (No. 10 infected with Malta strains and No. 2 infected with Catania strains) were transported to Catania and feeding experiments carried out. The result is shown in Table V.

TABLE V - FEEDING EXPERIMENTS WITH *P. perniciosus* FROM CATANIA

Species	Date fed	Total fed	Total positive	Remarks
	Chinese hamster No. 10 (Malta Strain)			
P. perniciosus	September 13	11	11	In 1 stomach only, after 3 days. In 3 others 6–7 days stomach only. In 7 cardia and stomach.
P. major	September 13	3	3	In 1 stomach only. In 1 heavy infection in stomach and in 5 flagellates in cardia. In 1 anterior part of cardia.
	Chinese hamster No. 2 (Catania Strain)			
P. perniciosus	September 9	31	31	In all anterior part of cardia.

As can be seen from the table the behaviour of *L. infantum* is the same in Catania as in Malta sandflies.

Dogs—Feeding experiments were carried out on three naturally infected dogs caught in Malta during 1931. The total number of sandflies infected was 280 and the infection rates on the three animals were 61%, 65% and 30% respectively. The histological changes in the skin of these animals were recorded in a previous paper.

As with sandflies infected with Maltese human strains of *L. infantum* from spermophils and Chinese hamsters there was a considerable variation in the distribution of flagellates in individual sandflies. Only one sandfly had a proboscis infection and no " short form " infections were seen.

Greek Strains of L. infantum

Chinese Hamster No. 12—Inoculated with Greek strains of *L. infantum* of canine origin. Between June 29 and July 21, 1931, 4 cc of culture inoculated intraperitoneally. Feeding experiments carried out on August 30 and September 2, 1931, gave positive results (23 out of 35 sandflies infected).

Cricetus auratus No. 1—Inoculated with a Greek human strain of *L. infantum*. On September 14 and 15, 1931, 80 sandflies fed and seven became infected.

The Greek strains resembled the Maltese in their behaviour in *P. perniciosus*, in that they showed a similar proportion of stomach infections.

Feeding Experiments in 1932

A review of the results during 1930 and 1931 showed several interesting facts. There was a very definite difference between the behaviour of the Malta and Catania strains of *L. infantum* in *P. perniciosus*. The latter gave almost uniform infections in the anterior part of the cardia while in the former the distribution of flagellates in the sandflies was variable. While it was impossible to distinguish between Malta and Catania strains by their behaviour in a small number of sandflies the examination of a large number of infected insects showed a very clear difference. In two successive years the majority of the " short form " infections and the proboscis infections occurred towards the end of the sandfly season. This was very striking in three animals (Chinese hamsters Nos. 1, 10 and 11). In Chinese hamster No. 2, five proboscis infections (out of 331 infected sandflies) were noted irregularly in the beginning and middle of the sandfly season, but as this animal died on September 13, 1931, the observation could not be continued till the end of the season.

It appeared unlikely that the preponderance of " short forms " and proboscis infections towards the end of the sandfly season was a mere coincidence. It was already found in 1930 and 1931 that sandflies *P. perniciosus* of various months were not physiologically identical and it was natural to correlate these two facts.

A number of factors had to be considered.

1. Variations in individual sandflies. This was proved definitely.
2. Variation of the protozoon in different hosts and the effect of duration of infection in a single host on the distribution of the flagellates in the sandfly.

It was therefore planned to carry out feeding experiments during 1932 on animals used during 1931 and to compare the results. Unfortunately it was not possible to proceed far with this work for Chinese hamster No. 10 died on April 4, 1932, and work with the other animals was cut short by an epidemic of Pasteurella introduced into the animal room in the quarantine station in Malta by an apparently healthy rabbit.

TABLE VI—FEEDING EXPERIMENTS IN 1932 ON ANIMALS INFECTED WITH MALTESE STRAINS DURING 1931

Species	Date	Total fed	Total positive	Remarks
		Chinese hamster No. 11		
P. perniciosus	May 24	1	1	Stomach and anterior part of cardia.
	,, 27	44	42	In 1 stomach only, in 2 stomach and lower part of cardia, in remainder up to anterior part of cardia.
	,, 29	36	36	In 3 stomach only, in 7 stomach and lower part of cardia, in remainder anterior part of cardia.
P. major	,, 29	7	7	In 2 stomach only, in 5 top of cardia.
		Chinese hamster No. 16		
P. perniciosus	June 10	56	55	In 4 stomach only, in 9 stomach and lower part of cardia.
	,, 11	48	44	In 1 stomach only, in 9 up to middle of cardia, in remainder top of cardia.
P. major	May 27	2	2	Stomach and anterior part of cardia.

As will be seen from Table VI the number of infections confined to the stomach was smaller than that found in the same animals in the middle of the sandfly season of 1931. In Chinese hamster No. 11 (the only one which allowed sufficient data for comparison) the number of anterior infections was smaller than that obtained from the same animal towards the end of the sandfly season 1931 and there were no proboscis and no "short form" infections. In both animals the infections were very heavy.

As no animals infected with Maltese strains survived the epidemic of Pasteurella, two more animals were infected and the feeding experiments

repeated, but as some time elapsed before a skin infection was developed, the greater part of the sandfly season was lost.

Chinese Hamster No. 74—Between June 9 and August 13, 1932, received 5 cc of culture of Maltese *L. infantum* intraperitoneally. Feeding experiments first gave positive results on August 17, 1932.

TABLE VII—SHOWING RESULTS OF FEEDING *P. perniciosus* ON CHINESE HAMSTER NO. 74

Date	Total fed	Total positive	Remarks
August 17 ..	11	2	In 1 stomach only, in 1 top of cardia.
September 13	7	2	Stomach and posterior half of cardia.
October 5 ..	25	6	In 2 stomach only, in remainder anterior part of cardia.
October 30 ..	11	6	In all anterior part of cardia.

Catania Strain

Feeding experiments were carried out on infected Chinese and Syrian hamsters. Owing to the epidemic of Pasteurella it was not possible to carry out an experiment on an individual animal throughout the whole sandfly season, but in one animal (Chinese hamster No. 14) an almost uninterrupted series of experiments was carried out from July 2 till September 3, 1932, with Maltese sandflies and from September 19 till November 5, 1932, with Catania sandflies and on November 18, 1932, again with Maltese sandflies.

Chinese Hamster No. 14—June 30, 1931, flagellates from the cardia of sandfly No. 192 were rubbed into a scarified point on the back. (Sandfly No. 192 fed June 26, 1931, on Chinese hamster No. 2. Dissected June 30, 1931. Stomach, cardia and œsophageal valve were infected with flagellates.) June 29, 1932, piece of spleen removed. Spleen enlarged and infected.

Feeding experiments on July 2, 1932, gave positive results. The animal died April 7, 1933.

Out of 523 *P. perniciosus* fed 504 were found positive; of this number only one sandfly had an infection confined to the stomach while in the remainder the anterior part of the cardia was uniformly infected. This is a convincing demonstration of the difference between Catania and Malta strains of *L. infantum*. The results were uniform except for the occurrence of " short forms " and proboscis infections as shown in Table VIII.

The following table clearly shows the increase of both " short forms " and proboscis infections towards the end of the sandfly season.

TABLE VIII—OCCURRENCE OF "SHORT FORM" AND PROBOSCIS INFECTIONS IN CHINESE HAMSTER NO. 14

Period	No. of infected sandflies dissected	No. of proboscis infections	No. of "short form" infections
Maltese Sandflies			
July 2 to September 3	114	0	0
November 18	4	1	2
Catania Sandflies			
September 19 to September 26.	50	0	0
September 27 to October 5	63	1	1
October 6 to October 13	86	3	8
October 14 to October 21	56	5	9
October 22 to October 29	82	4	7
October 30 to November 4	29	3	3

Chinese Hamster No. 47—Inoculated intraperitoneally October 29, 1931, with liver from Chinese hamster No. 26. February 22, 1932, piece of spleen removed and found infected. Between May 31 and June 5, 1932, 19 *P. perniciosus* fed and 18 became infected. In all the infection reached the anterior part of the cardia.

Chinese Hamster No. 50—September 21, 1931, *P. perniciosus* No. 1288 infected with Maltese *L. infantum* laid eggs and re-fed on this animal. Sandfly died on September 28 and was found to contain flagellates in the pharynx.

July 1, 1932, a piece of spleen was removed and found negative. As very few animals survived the epidemic of Pasteurella we were obliged to re-inoculate this animal. Between July 15 and 21, 1932, flagellates from nine sandflies infected with Catania strains by feeding on Syrian hamsters were inoculated intraperitoneally. Between August 15 and September 1, 1932, 3 cc of culture were inoculated intraperitoneally.

September 11, 1932—Eight sandflies fed, of which one was positive after five days. The flagellates were confined to the stomach. October 5, 1932, six sandflies fed, of which one was positive after six days. The infection was confined to the stomach.

The number of infected sandflies was very small, but the result is of interest since two stomach infections occurred in sandflies fed on an animal inoculated with Catania strains.

Chinese Hamster No. 51—Inoculated intraperitoneally November 3 and 4, 1931, with flagellates from seven infected sandflies. June 10,

1932, 10 *P. perniciosus* fed on this animal and one became infected. Flagellates were numerous in the anterior part of the cardia.

Chinese Hamster No. 52—November 5, 1932, inoculated intraperitoneally with flagellates from four sandflies infected with Catania strains of *L. infantum*. On June 16, 1932, and September 19, 1932, 28 *P. perniciosus* fed on this animal and 27 became infected. All contained flagellates in the anterior part of the cardia. One had a proboscis infection and none had " short forms."

Chinese Hamster No. 61—Between March 24 and April 15, 1932, received 4 cc of culture intraperitoneally. June 23, 1932, seven sandflies fed on this animal and one was positive after five days. The infection was slight but the flagellates reached the anterior part of the cardia.

Cricetus auratus—Between June 20 and August 16, 1932, 447 sandflies fed on five Syrian hamsters infected with Catania strains. 325 (dissected four days or more after feeding) were subsequently found positive. All five animals gave similar results. The distribution of the flagellates in the sandflies was as follows: in 302 sandflies the flagellates reached the anterior part of the cardia, in 15 up to the middle of the cardia, in one up to the lower part of the cardia, and in seven the infection was confined to the stomach. The tendency to adopt an anterior position is rather less in sandflies fed on Syrian than in those fed on Chinese hamsters.

Dogs—During 1932 feeding experiments with *P. perniciosus* were carried out on 13 naturally infected dogs in all stages of the disease with the object of determining the influence of the vertebrate host on the course of the infection in the sandflies. Between May 23 and November 12 (1932), 783 infected sandflies were dissected and the distribution of flagellates studied. The infection rates in the various animals varied from 5% to 100%. In individual animals the skin infection changed progressively and the infection rate rose from 0% to 70% within four months as shown in a previous paper. The same irregularity in the distribution of flagellates was noted as in the previous year. During the greater part of the sandfly season the flagellates were confined to the stomach in 25% to 30% of infected sandflies (as in the case of sandflies infected on spermophils) but towards the end of the season the percentage fell to 8% or less. Out of 783 sandflies only 3 had " short form " infections and 4 has proboscis infections (2 out of the " short form " infections). During 1931 and 1932 out of a total of 1063 sandflies infected on dogs only 5 had proboscis infections. All the " short form " and proboscis

infections occurred towards the end of the sandfly season between October 9 and November 8, 1932 (out of 597 infected during period).

There is a marked influence of the host on the Leishmania for the proportion of "short form" and proboscis infections is much smaller in sandflies infected on dogs than on those infected on Chinese hamsters.

Three naturally infected dogs used for feeding experiments in Malta were transported to Jerusalem and laboratory bred sandflies were allowed to feed on them. The results of these feeding experiments can hardly be compared to the previous ones for the sandflies were obtained by forcing the development of hibernating larvæ at a temperature of 30° C. A total of 87 sandflies fed and of these 64 became infected. In 16 sandflies the infection was confined to the stomach. The proportion of purely stomach infections was considerably more than that found in the same animals towards the end of the sandfly season of 1932 but this point cannot be stressed for the numbers were few and the sandflies used cannot be considered as normal.

Feeding Experiments on an Experimentally Infected Dog

The animal used was caught in Malta and inoculated intrahepatically with a human Catania strain of *L. infantum* (see previous paper). Before the inoculation the spleen was examined and found negative. As previously shown a negative spleen examination does not necessarily mean that an animal is free from infection but the histological changes in the liver indicated that the infection probably followed the inoculation. Out of 29 sandflies fed on April 5 and April 9, 1932, 13 became infected and in 8 of these the infection was confined to the stomach.

Feeding Experiments on Cases of Infantile Leishmaniasis

During 1931 and 1932, 176 sandflies (*P. perniciosus*) were fed on five clinically severe cases of infantile Kala Azar all showing numerous L.D. bodies in spleen smears, and only three sandflies became infected. This low infection rate is negligible as compared to that obtained in experiments on dogs and other animals.

Natural Infection in P. perniciosus

Four out of 150 *P. perniciosus* collected in the municipal dog house were found infected with *L. infantum*, while all *P. papatasii* examined from this place were found to be negative. The positive findings in *P. perniciosus* are only to be expected in view of the fact that a large number

of dogs are always kept in the dog house and 11% of dogs in Malta are infected with *L. infantum*.

Infection of P. perniciosus *with* L. donovani (*India*)

On September 9 and 13, 1931, 20 sandflies fed on a Syrian hamster infected with an Indian strain of *L. donovani*. Four days later flagellates were found in six sandflies and in five they were confined to the stomach.

The Behaviour of L. infantum *in Sandflies other than* P. perniciosus

1—*P. papatasii*—The behaviour of several strains of *L. infantum* and *L. donovani* in *P. papatasii* has been previously discussed (1917 and 1931). It was found that cultures of Naples strains of *L. infantum*, when ingested by *P. papatasii*, produce a low infection rate, but the flagellates attach themselves to the anterior part of the cardia. This was later confirmed by the feeding experiments on an infected Chinese hamster in Catania. One Mediterranean human strain of *L. infantum* studied in 1927 was quite different from the Italian strains in that the bulk of the infections were limited to the stomach. The difference between the latter strain and those from Naples and Catania was quite distinct. (The infection rate produced by two Indian strains of *L. donovani* in *P. papatasii* was higher than that produced by *L. infantum* from Naples, but the infection was confined to the stomach in 75% of the infected sandflies.)

During 1931 and 1932 many *P. papatasii* were fed on naturally infected dogs with a negative result. Feeding experiments were repeated in 1933 on three naturally infected dogs which were brought from Malta to Jerusalem in November, 1932. By February, 1933, the skin infection had become so intense that it was possible to infect *P. papatasii* on these animals.

117 sandflies fed on one animal and 24 became infected. In 7 out of 15 sandflies dissected after four days the infection was confined to the stomach (the infection rate in *P. perniciosus* was 91%).

104 sandflies fed on the second animal and seven became infected. In six the infection was confined to the stomach (infection rate in *P. perniciosus* 90%).

115 sandflies fed on the third animal and 15 became infected. In seven the infection was confined to the stomach (infection rate in *P. perniciosus* 98% in November, 1932).

As in *P. perniciosus*, the canine strains of *L. infantum* from Malta produce a large proportion of purely stomach infections in *P. papatasii*, in contra distinction to the human strains from Naples and Catania.

Four out of seven *P. sergenti* became infected by feeding on animals with an intense skin infection.

2. *P. major.* The behaviour of Catania strains of *L. infantum* in *P. major* has already been reported in 1931. The flagellates uniformly invade the anterior part of the cardia. The number of feeding experiments with *P. major* on Maltese human and canine strains were few because this sandfly is comparatively rare in Malta.

Between September 22 and October 2, 1932, 11 sandflies were fed on a naturally infected dog and 10 became infected. In four sandflies the infection was confined to the stomach. (The infection rate in *P. perniciosus* during this period was 70%.) The number of *P. major* fed on human strains from Malta is insignificant, but the results indicate that as in *P. perniciosus* there is a considerable proportion of purely stomach infections.

In view of the absence of *P. perniciosus* and the prevalence of *P. major* in Greek foci of infantile Kala Azar it was obviously desirable to carry out simultaneous feeding experiments on infected animals with both sandflies. For this purpose a naturally infected dog was transported from Malta to Catania where *P. major* was found to be numerous in the limited area during the sandfly season of 1930. A house was chosen in which 50 to 100 unfed females could be caught in an evening during the sandfly season of 1930. It was found that *P. major* was not as common in 1932, for only 5 to 20 unfed females per evening were found in the same house between August 21 and September 21. Between August 21 and August 29, 1932, 31 *P. major* and 119 *P. perniciosus* fed on the animal; 25 *P. major* and 31 *P. perniciosus* became infected. On September 18, 1932, 10 *P. major* and 20 *P. perniciosus* fed on the same animal; of the former all were infected and of the latter only 15. It is thus evident that the infection rate in *P. major* is higher than in *P. perniciosus*.

The above experiment has still another significance. Both species take almost equal feeds. It is therefore reasonable to suppose that the same percentage of both species fed at the same time on the same animal ingest infected cells. Nevertheless, the infection rate in *P. perniciosus* was only 28·6% while that of *P. major* was 80%, *i.e.*, at least 80% of *P. perniciosus* ingested infected cells and only 28·6% became infected. The number of parasites ingested by both species was small, for it was shown previously that when the infection rate in *P. perniciosus* is about 30% not only are the infected cells in the skin of the dog relatively few, but each cell has relatively few parasites (usually two or three). It is therefore evident that *P. perniciosus* is to some extent resistant to small

doses of *L. infantum* but not to large ones. A similar phenomenon has been previously recorded in the case of *P. papatasii* which is readily infected by small doses of Palestinian *L. tropica*, but is resistant to relatively high doses of Baghdad strains (2500 flagellates per cmm). This resistance is partly broken down by very high doses (10,000 flagellates per mm^3).

Infection of P. sergenti *and* P. major *with* L. tropica

Between June 27 and August 8, 1931, 14 sandflies (five *P. seregnti*, six *P. major* and three *P. papatasii*) caught in Malta were fed on an experimental Oriental sore (Baghdad strain).* This strain had been previously investigated and found to give a low infection rate in *P. papatasii* in which the flagellates when established invariably adopted an anterior position.

Four *P. sergenti* and two *P. major* became infected; in the former the flagellates ascended to the anterior part of the cardia while in the latter they were confined to the stomach. Both species of sandflies were too rare to continue this experiment profitably in Malta.

Infection of other Sandflies of the Major *Group with* L. infantum *and* L. donovani

The experiments recorded below were carried out between September 18 and October 15, 1933, in Palestine. Wild sandflies of the following species were used: (1) *P. macedonicus*, (2) *P. major* var. *syriacus*, (3) *P. perniciosus* var. *tobbi*, (4) *P. chinensis* var.

None of the above sandflies can be regarded as good carriers of infantile Leishmaniasis for very few cases occur in localities where they are common. *P. macedonicus* is common in the plain of Esdraelon where only two cases have occurred during the last six years. *P. major* var. *syriacus* occurs in parts of Jerusalem where only one locally acquired case was found during the last ten years, while *P. major* var. *syriacus*, *P. perniciosus* var. *tobbi* and *P. chinensis* are common in upper Galilee where the disease is practically unknown.

Three Syrian hamsters were employed, one inoculated with an Indian strain of *L. donovani*, one with a Maltese strain of *L. infantum*, and one with a Catania strain of *L. infantum*.

The results are shown in Table IX.

* Produced by direct inoculation on October 28, 1929. This volunteer had been previously found refractory to Palestinian *L. tropica* after repeated inoculation of flagellates from infected sandflies.

The number of sandflies fed is too small to draw strict conclusions but there is a clear indication of variation in individual sandflies of all the four species.

The Effect of Re-feeding on the Distribution of Flagellates

The distribution of flagellates was observed in 52 infected *P. perniciosus* which re-fed on normal dogs. These sandflies lived from 6 to 17 days after the infecting feed and the distribution of flagellates was the same as in sandflies dissected four days and more after the infecting feed. Actually the most advanced anterior infections were found in sandflies dissected four or five days after the first feed. It is therefore evident that re-feeding has no effect on the distribution of flagellates.

TABLE IX

Strain	Species of sandfly	No. fed	No. positive	Remarks
L. donovani-India ..	*macedonicus*	9	6	In 1 stomach only after 4 days.
,, ..	*major* var. *syriacus*	34	25	In 8 stomach only after 4 to 8 days.
,, ..	*chinensis*	22	19	In 4 stomach only after 7 days.
L. infantum-Catania	*major* var. *syriacus*	14	2	Both cardia and stomach.
,,	*chinensis*	1	1	Stomach only after 9 days.
,,	*papatasii*	12	1	Stomach only after 9 days.
L. infantum-Malta	*major* var. *syriacus*	9	5	All in cardia and stomach.
,,	*chinensis*	18	13	In 5 stomach only after 6 days.
,,	*perniciosus* var. *tobbi*	6	6	In 1 stomach only after 6 days.

Attempts to Transmit L. infantum *per os*

October 8, 1931 two Chinese hamsters were fed on heavily infected liver of a dog caught in Malta. Both animals died of Pasteurella (September 6 and 14, 1932) and were found negative for Leishmania.

Mode of Transmission of L. infantum

It has already been pointed out that in the case of infants the only mode of transmission which need be taken into consideration is one which does not involve active participation on the part of the vertebrate host *i.e.*, transmission by bite. It was necessary to determine (*a*) whether flagellates introduced into the skin produce infection, and (*b*) the conditions under which flagellates from infected sandflies enter the skin.

Inoculation of Flagellates into the Skin of Susceptible Animals

In each case a small incision was made into the skin and flagellates rubbed into the wound. The incision did not go through the subcutaneous tissue.

Chinese Hamster No. 14—June 30, 1931—Inoculated into the skin with flagellates (long forms) from the cardia of sandfly No. 192. June 29, 1932—The spleen was found infected.

Chinese Hamster No. 15—July 1, 1931—Inoculated into the skin with flagellates (long forms) from an infected sandfly. · June 14, 1932 – died of Pasteurella. Spleen negative.

Chinese Hamster No. 35—September 9, 1931 –Inoculated into the skin with flagellates (short forms) from two infected sandflies. June 11, 1932, died of Pasteurella. Spleen contained numerous L.D. bodies.

In the above three experiments only a very small proportion of the flagellates from the infected sandflies were used but the number was not determined.

Spermophil No. 30—August 18, 1932—Inoculated into the skin with flagellates from an infected sandfly. November 20, 1933 spleen examined and found heavily infected. This animal survived for a much longer period than other spermophils inoculated intraperitoneally. Evidently the number of flagellates introduced influences the length of the incubation period and the course of the disease.

The Exit of Flagellates from Infected Sandflies

Since relatively few sandflies re-fed on animals the conditions of exit of flagellates from infected sandflies were studied in a Hertig apparatus. It was previously shown (1931) that infections of the pharynx and buccal cavity in *P. perniciosus* are not in themselves sufficient to ensure the exit of flagellates in a Hertig apparatus.

In the earlier experiments sandflies were placed in the Hertig apparatus after light narcotization with ether. It was thought that the ether might affect the movement of the flagellates and in the experiments carried out in 1931 and 1932 the sandflies were either lightly stunned in test tube or immobilized by moistening their wings. The insects quickly recovered from the effects of the stunning and behaved like normal sandflies.

During 1931 and 1932, 52 heavily infected *P. perniciosus* were placed in a Hertig apparatus and flagellates were obtained only from two which were both found to be infected in the proboscis. Four sandflies in which

flagellates were found in the proximal part of the epipharynx did not inject flagellates into the capillary.

The numbers of flagellates deposited by the two sandflies were approximately 1200 short forms and 60 long and short forms respectively. In one case about 2000 flagellates (short forms) were seen in the distal part of the proboscis of a freshly dissected sandfly four days after the infecting feed.

The above results confirm the previous findings and give additional support to the view that flagellates emerge from an infected sandfly *only* if the distal part of the proboscis is infected.

A study of the biting and pumping apparatus of sandflies also supports the view that the sandfly cannot actively eject flagellates. In sections of whole infected sandflies both the pharynx and buccal cavity may be found filled with flagellates. This does not imply blocking of these channels for though they are full of flagellates in the resting condition the powerful dilator muscles of the pharynx and buccal cavity when in action are capable of increasing their volume sufficiently to allow the ingress of blood during the act of feeding. The contractions of these muscles create a negative pressure which produces the flow of blood from the wound into the food canal. There is no special muscular apparatus for ejecting fluid or flagellates through the part of the food canal which lies in the proboscis (*i.e.*, between the lower surface of the epipharynx and the upper surface of one mandible) into the wound. It is also hardly probable that flagellates enter the wound during the second part of the act of feeding (after the preparatory interval) when there is already a current of blood passing up the proboscis, for the average flow of blood through this part is about 2300 μ per second, while the maximum velocity of an active Leishmania is only about 20 μ per second.* Flagellates can enter the wound during the preparatory interval from the distal part of the epipharynx either by their own activity or by being *passively deposited into the tissue fluids during the movements of the epipharynx in the tissues.* That the latter actually occurs is proved conclusively by the fact that some of the flagellates found in the capillary of the Hertig apparatus are sluggish and incapable of active translatory movement.

* The mechanics of the flow of blood through the proboscis are rather complicated. The average width of the food canal in the proboscis is about 20 μ and the mean velocity of blood through this part of the food canal is about 2300 μ per second. The velocity near the sides would naturally be expected to be much less. It is difficult to determine the width of the zone at the sides in which the velocity of fluid is less than that of an active flagellate (maximum velocity about 20 μ per second). The pressure of the tissue fluid at the site of the bite has also to be taken into account.

Attempts to Transmit by Bite

During 1931 and 1932 attempts were made to transmit *L. infantum* by bite to one Chinese hamster and three dogs. Chinese hamsters are unsuitable for re-feeding experiments with *P. perniciosus* for this sandfly may wait for hours before feeding on hamster and it was found that hamsters (and spermophils) frequently destroy and swallow sandflies which attempt to feed on them. There was therefore a very serious possibility of infecting a hamster with sandflies per os and the experiments were discontinued.

September 13, 1931—Sandfly No. 1288 infected by feeding on an infected hamster. The sandfly laid eggs and re-fed on Chinese hamster No. 50 on September 21, 1931. The sandfly was dissected on September 28, 1931. Numerous flagellates were found in the pharynx. July 1, 1932, spleen of hamster No. 50 examined and found negative.

Infected sandflies re-fed on two dogs during 1931 and 1932, but as none of these insects had a sufficiently anterior infection it was not considered worth while to keep the animals under observation.

Between June 15 and October 24, 1932, 24 infected sandflies re-fed on a dog imported from England. All were subsequently found to have infections extending beyond the anterior part of the cardia, in two the pharynx was infected and in another two the infection extended *into the proximal part of the epipharynx.*

March 25, 1933—The animal died of pneumonia. No L.D. bodies were found in spleen, liver or bone marrow.

Infection by Flagellates Deposited from the Proboscis of a Single Sandfly

October 15, 1932—*P. perniciosus* No. 694 fed on Chinese hamster No. 14. October 20, 1932—sandfly placed in a Hertig apparatus and the fluid from the capillary examined. About 1200 flagellates (short forms) were found. The fluid was inoculated through a fine capillary into the skin of spermophils Nos. 31 and 32. It is obvious that in these manipulations of 1 to 2 cmm of fluid many of the flagellates were inevitably lost.

Result—March 28, 1933—spermophil No. 31 died. The spleen was not enlarged. A few L.D. bodies were found in smears of spleen and liver. January 28, 1933—spermophil No. 32 died. Spleen and liver negative.

October 20, 1932—the proboscis of sandfly No. 694 was dissected off and introduced into a small incision on the back of spermophil No. 33. November 3, 1933—No. 33 died. Spleen and liver negative.

November 18, 1932—*P. perniciosus* No. 2065 fed on Chinese hamster No. 14. The sandfly was transported to Jerusalem in a small cage kept in a moist chamber. November 25, 1932—the sandfly was placed in a Hertig apparatus and about 60 flagellates (long and short forms) were recovered. The flagellates were inoculated into the skin of spermophil No. 34.

February 4, 1933—spermophil No. 34 died. Spleen and liver negative. The positive result obtained with spermophil No. 31 is the nearest approach to transmission by bite.

Discussion

All the available experimental evidence implies that only those infected sandflies which have flagellates in the distal part of the proboscis are capable of depositing flagellates in the skin during the act of biting. All other infected sandflies including those in which pharynx and buccal cavity are invaded are incapable of transmitting by bite and are harmless from the point of view of transmission to infants. It has been shown by dissections on a large scale that the overwhelming majority of infected sandflies are not infected in the proboscis and are therefore incapable of transmission by bite.

Sandflies *P. perniciosus* (the species which has been studied most intensively) of various generations although morphologically indistinguishable, differ physiologically. This fact has been demonstrated beyond doubt by observations during three consecutive years. There are two distinct strains of Mediterranean *L. infantum*, Malta and Catania. There are also at least two distinct strains of *L. tropica*, a Baghdad and Palestinian. The difference between Baghdad and Palestinian strains of *L. tropica* is very striking and remains constant after eight years culture on artificial media. They can be distinguished by their infection rates in *P. papatasii* even if they are inoculated into and cultured from the same individual host. It is interesting to note that a volunteer successfully inoculated on September 9, 1925, with a Palestinian strain of *L. tropica* from which he recovered spontaneously was infected experimentally with a Baghdad strain on October 28, 1929. Both strains cultured from this volunteer could easily be distinguished by their infection rate in *P. papatasii*. There is apparently no rigid cross immunity in human beings between Baghdad and Palestinian *L. tropica*. It is highly probable that there are distinct strains within all the recognized species of human Leishmania. The Malta strain produces a large percentage of infections confined to the stomach during the greater part of the sandfly season but *towards the end of the season from the end of September to the middle of*

November the infections become more anterior and a small number of proboscis infections appear. The latter are very few and with sandflies infected on dogs are less than 1% of the total number of infected insects. Catania strains produce a negligible percentage of infections confined to the stomach; throughout the whole of the sandfly season the anterior part of the cardia is invaded and a few proboscis infections appear sporadically throughout the season but *as in the case of the Malta strains the majority of proboscis infections appear towards the end of the sandfly season.*

These findings imply that the vast majority of cases acquire the infection towards the end of the sandfly season and that *P. perniciosus* is not very dangerous throughout most of the sandfly season. It is interesting to note that though cases of infantile Leishmaniasis occur throughout the whole year they are most prevalent between the end of April to the middle of July, *i.e.*, six to eight months after the end of the sandfly season. In this relatively short period fully 50% of the total number of cases appear for diagnosis. In other words at least 50% of the cases appear six to eight months after the period of maximum prevalence of proboscis infections in sandflies.

The reason for the difficulty of experimental transmission by bite is obvious. The numbers of infected sandflies with flagellates in the distal part of the proboscis is so small that it would require re-feeding enormous numbers of infected sandflies in order to be certain of including one with a suitable infection, and a positive result after a relatively small number of re-feeds would be merely a matter of chance. Unfortunately wild sandflies after laying eggs in the laboratory behave similarly to laboratory bred sandflies and only a small proportion re-feed. It was not practical to work with laboratory bred sandflies since for some undetermined reason very few of them feed although they appear normal in every way.

L. infantum may be described as being incompletely adapted to *P. perniciosus*. This is manifested by the variation in the type of infection in individual sandflies and by the scarcity of proboscis infections. In the vast majority of cases ingestion by a sandfly is the termination of the life-cycle of the protozoon which in spite of rapid multiplication cannot enter a new host.

The large proportion of infected dogs and the constant occurrence of relatively few new cases of infantile Kala Azar (250 per annum in a population of 250,000 in Catania) in spite of the low incidence of proboscis infections is readily explained by the enormous numbers of *P. perniciosus* in endemic areas. Both in Catania and Malta this species is the commonest of all biting insects which come into contact with man and in view of

their prodigious numbers it is surprising that the number of cases is not greater.

In appraising the efficiency of a sandfly as a transmitter of infantile Leishmaniasis the following factors have to be taken into account.

1. The number of sandflies with proboscis infections. The infection rate in a sandfly although significant is not in itself a sufficient index of capacity to transmit by bite.

2. The prevalence of reservoirs capable of infecting sandflies. In the Mediterranean this role is played by dogs and the number of sandflies which infect themselves on human cases is comparatively small.

3. The density of the sandfly population.

Summary and Conclusions

Phlebotomus perniciosus and *P. major* were infected with *L. infantum* by feeding on dogs, hamsters and spermophils and the distribution of flagellates in the sandflies was studied.

P. perniciosus was infected by feeding on human cases of infantile Leishmaniasis.

The experimental infection rate with *L. infantum* is higher in *P. major* than in *P. perniciosus.*

The following species of the major group were also infected with *L. infantum* by feeding on animals: *P. major* var. *syriacus*, *P. perniciosus* var. *tobbi*, *P. chinensis* var.

P. papatasii and *P. sergenti* can be infected with *L. infantum* by feeding on animals with a very intense infection.

P. perniciosus, *P. perniciosus* var. *tobbi*, *P. major* var. *syriacus*, *P. chinensis* var. *and P. macedonicus* were infected with Indian strains of *L. donovani* by feeding on hamsters.

There is a considerable variation in the distribution of flagellates in individual sandflies.

There is a distinct difference between the behaviour of Maltese and Catanian strains of *L. infantum* in *P. perniciosus.* The Maltese strains produce a large percentage (up to 30%) of purely stomach infections during the greater part of the sandfly season. The Catanian strains except in a negligible number of cases invade the anterior part of the cardia. In the Maltese strains the infections in the sandfly become more anterior towards the end of the sandfly season.

Infections of the proboscis are very rare in sandflies infected with both Maltese and Catanian strains.

The majority of proboscis and short form infections occur towards the end of the sandfly season.

Only sandflies with an infection in the distal part of the proboscis are capable of transmitting by bite and therefore it is inferred that the majority of infections in human beings are acquired towards the end of the sandfly season. This view is supported by the fact that at least half of all cases in Catania are diagnosed from the end of April to the middle of July, *i.e.*, from six to eight months after the end of the sandfly season.

Susceptible animals can be infected by the inoculation of flagellates into the skin.

A spermophil was infected by introducing flagellates deposited from the proboscis of an infected sandfly into the capillary of a Hertig apparatus. This corresponds to transmission by bite.

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Investigations on Mediterranean Kala Azar

X A Note on *Trypanosoma platydactyli* and *Leishmania tarentolæ*

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(*Communicated by Sir Henry Dale, Sec. R.S.—Received June* 18, 1934)

The presence of *Trypanosoma platydactyli** was previously recorded in the gecko *Tarentola mauretanica* from Catania (Adler and Theodor (1931)), where 17 out of 49 specimens were found infected. This trypanosome was also found in Malta during 1931 and 1932 in 6 out of 9 geckoes examined. It was found that it is not necessary to examine geckoes by culture from the heart's blood, for a diagnosis can be established in three days by feeding *Phlebotomus parroti* or even *P. papatasii* on a gecko. Forty out of 43 *P. parroti* which fed on nine infected geckoes became infected with the trypanosome. The flagellates adopt an anterior position and reach up to the œsophagus in three to four days. In no case was a proboscis infection found during an observation period of nine days and not a single instance of infection in the hindgut was found.

In one instance a gecko apparently infected itself by swallowing an infected *P. parroti*, but infection by bite is not excluded.

Gecko No. 7—July 4, 1931—Cultures from the heart's blood negative during three weeks observation. July 15, 1931—Two sandflies which had previously fed (July 11, 1931) on gecko No. 8 infected both with *L. tarentolæ* and *T. platydactyli* re-fed on gecko No. 7. The re-feeding experiments were carried out in a tightly closed Erlenmeyer flask. One of the sandflies disappeared and was apparently eaten by the gecko. It is to be noted that *T. mauretanica* does not appear to ingest unfed *P. parroti*, but on several occasions during 1931 and 1932 gorged *P. parroti* disappeared when kept together with the gecko in a closed Erlenmeyer flask and it was quite clear that they were not crushed, because crushed sandflies (particularly after gorging) can always be detected.

* Erroneously referred to as *T. ptyodactyli* in a previous paper ('Proc. Roy. Soc.,' B, vol. 108, pp. 492–3).

August 15, 1931—One *P. parroti* fed on gecko No. 7. August 24, 1931—The sandfly died. On dissection stomach and cardia were found choked with trypanosomes. Subsequently between August 16 and September 26, 1931, seven out of eight *P. parroti* infected themselves by feeding on this gecko. The inference is that gecko No. 7 infected itself with *T. platydactyli* by swallowing an infected sandfly. In this case transmission by bite is not very probable though not absolutely excluded.

It was previously noted that the strain of *T. platydactyli* may lose its motility and remain with only a rudimentary flagellum after a number of subcultures on Locke Serum Agar. This is apparently the rule for two other strains of *T. platydactyli* isolated from naturally infected geckoes in Malta also lost their motility and now possess only a rudimentary flagellum.

One gecko, *T. mauretanica* No. 8 was found to have a mixed infection of *T. platydactyli* and *L. tarentolæ*. The diagnosis was established both by culture and by feeding experiments with *P. parroti*. Three sandflies fed on July 11, 1931, on the gecko were dissected on July 15, 21, and 23, respectively, and were found infected both with the trypanosome and Leishmania.

L. tarentolæ was found in the stomach and anterior part of the cardia. The flagellates were active and of the short form type. *L. tarentolæ* is not sufficiently common in Malta to pursue this investigation profitably and it is obvious that *P. parroti* is not the best vector of *L. tarentolæ*. The latter is to be sought in places such as Tunis where 36% of geckoes are infected and sandflies of the minutus group other than *P. parroti* occur.

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